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(54) Title: MITOCHONDRIAL DNA MUTATIONS THAT SEGREGATE WITH LATE ONSET DIABETES MELLITUS (57) Abstract <p>The present invention relates to genetic mutations in mitochondrial genes that segregate with diabetes mellitus. The invention provides methods for detecting such mutations, as a diagnostic for diabetes mellitus, either before or after the onset of clinical symptoms. Examples of specific mutations in the mitochondrial ATP synthase 8/6 gene and tRNA^{Lys} gene are given. The invention also provides treatments for dysfunctions due to mitochondrial genes that segregate with diabetes mellitus. Cybrid cell lines are described which are useful as model systems for the study of the mitochondrial metabolic disorders that are associated with diabetes mellitus, and for identifying therapeutic compounds and treatments for this disease.</p>		

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MITOCHONDRIAL DNA MUTATIONS THAT SEGREGATE WITH LATE ONSET DIABETES MELLITUS

TECHNICAL FIELD

The present invention relates generally to model systems for diseases
5 that involve defects in the function of mitochondria, where those defects arise from
defects in the genes of those mitochondria. The invention also relates to the use of
these model systems for screening drugs and evaluating the efficacy of treatments for
those diseases. In particular, the invention relates to the diagnosis and treatment of late
onset diabetes mellitus and related pathologies, such as impaired glucose tolerance and
10 insulin dependent or non-insulin dependent diabetes.

BACKGROUND OF THE INVENTION

Diabetes mellitus is a common degenerative disease affecting 5 to 10
percent of the population in developed countries. It is a heterogenous disorder with a
strong genetic component; monozygotic twins are highly concordant and there is a high
15 incidence of the disease among first degree relatives of affected individuals. The
propensity for developing diabetes mellitus is reportedly maternally inherited,
suggesting a mitochondrial genetic involvement. (Alcolado, J.C. and Alcolado, R., *Br.
Med. J.* 302:1178-1180 (1991); Reny, S.L., *International J. Epidem.* 23:886-890
(1994)).

20 Studies have shown that diabetes mellitus may be preceded by or
associated with certain related disorders. For example, it is estimated that forty million
individuals in the U.S. suffer from late onset impaired glucose tolerance (IGT). IGT
patients fail to respond to glucose with increased insulin secretion. A small percentage
of IGT individuals (5-10%) progress to insulin deficient non-insulin dependent diabetes
25 (NIDDM) each year. Some of these individuals further progress to insulin dependent
diabetes mellitus (IDDM). This form of NIDDM or IDDM is associated with decreased
release of insulin by pancreatic beta cells and/or a decreased end-organ response to
insulin. Other symptoms of diabetes mellitus and conditions that precede or are

associated with diabetes mellitus include: obesity, vascular pathologies, peripheral and sensory neuropathies, blindness, and deafness.

Due to the strong genetic component of diabetes mellitus, the nuclear genome has been the main focus of the search for causative genetic mutations.

5 However, despite intense effort, nuclear genes that segregate with diabetes mellitus are known only for rare mutations in the insulin gene, the insulin receptor gene, the adenosine deaminase gene and the glucokinase gene.

Clearly, a reliable diagnosis of late onset diabetes at its earliest stages is critical for efficient and effective intercession and treatment of this debilitating disease.

10 There is a need for a non-invasive diagnostic assay that is reliable at or before the earliest manifestations of late onset diabetes symptoms. There is also a need for developing therapeutic regimens or drugs for treating both the symptoms of diabetes mellitus and of the disease itself.

The present invention satisfies these needs for a useful diagnostic and
15 effective treatment of late onset diabetes and provides related advantages, as well.

SUMMARY OF THE INVENTION

The present invention relates to the identification of genetic mutations in mitochondrial genes, which segregate with late onset diabetes. The invention provides methods for detecting such mutations as a diagnostic for late onset diabetes, either
20 before or after the onset of clinical symptoms. More specifically, the present invention provides a method for detecting the presence or risk of developing diabetes mellitus in a human by determining the presence in a biological sample from a human of at least one mitochondrial mutation in an ATP synthase gene or in a tRNA-lysine (tRNA^{Lys}) gene that correlates with the presence of or risk of developing diabetes mellitus. The
25 invention further provides, but need not be limited to, particular nucleotide positions within ATP synthase gene sequences and in tRNA^{Lys} gene sequences where mutations that are silent mutations, missense mutations, or combinations thereof, may occur.

In one embodiment, the invention provides a method for detecting the presence or risk of developing diabetes by determining the presence in a biological

sample of at least one mitochondrial mutation that correlates with the presence or risk of developing diabetes mellitus. In various embodiments, the mutation is determined by hybridization with oligonucleotide probes, by ligation reaction, by polymerase chain reaction and variations thereof, or by single nucleotide primer-guided extension.

5 In another embodiment, the invention provides a method of detecting genetic mutations that cause diabetes mellitus or indicate a predisposition to develop diabetes mellitus by determining the sequence of at least one mitochondrial ATP synthase gene from humans known to have diabetes mellitus and comparing that sequence to the sequence of the corresponding wildtype mitochondrial ATP synthase
10 gene and identifying mutations in the humans known to have diabetes that correlate with the presence of the disease.

 The present invention also provides an isolated nucleotide sequence that is at least partially complementary to a mitochondrial DNA sequence, wherein the isolated sequence contains at least one mutation in an ATP synthase subunit 8/6 gene or
15 in a tRNA^{Lys} gene that correlates with the presence or risk of diabetes mellitus. The invention further provides an isolated nucleotide sequence that is at least partially complementary to a mitochondrial DNA sequence and that contains at least one mutation located between mitochondrial DNA nucleotides 8295 and 8571 that correlates with the presence or risk of diabetes mellitus. In certain aspects these isolated
20 nucleotide sequences are labeled with a detectable agent.

 In another embodiment, the invention provides a method of inhibiting the transcription or translation of one or more mutant ATP synthase encoding nucleic acids or transcription of one or more mutant tRNA^{Lys} encoding nucleic acids correlated with late onset diabetes mellitus, by contacting the ATP synthase or tRNA^{Lys} encoding
25 nucleic acids with antisense sequences specific to the mutant nucleic acids, and allowing hybridization between target mutant ATP synthase or tRNA^{Lys} encoding nucleic acids and the antisense sequences. Within preferred embodiments, hybridization is performed under conditions wherein the antisense sequences bind to and inhibit transcription or translation of target mutant ATP synthase 8/6 encoding
30 nucleic acid or inhibit transcription of target mutant tRNA^{Lys} encoding nucleic acid,

without preventing transcription or translation of the corresponding wild-type nucleic acids or other mitochondrial genes. Within certain embodiments, the mutant ATP synthase or tRNA^{Lys} encoding nucleic acids are RNA.

Within another aspect, a method for evaluating a compound for use in
5 diagnosis or treatment of diabetes mellitus is provided, comprising first contacting a predetermined quantity of the compound with cultured cybrid cells having genomic DNA originating from a ρ^0 cell line and mutant mitochondrial DNA originating from tissue of a human having a disorder that is associated with late onset diabetes mellitus, measuring a mitochondrial complex V activity that is affected by the mutant
10 mitochondrial DNA in the cybrid cells, and correlating a change in the mitochondrial complex V activity with effectiveness of the compound. Within certain embodiments the ρ^0 cell line that is immortal.

In yet another aspect, the invention provides a method for evaluating a compound for its utility in the diagnosis and treatment of diabetes mellitus. The
15 method generally comprises inducing differentiation of cultured undifferentiated cybrid cells having genomic DNA originating from a ρ^0 cell line and mutant mitochondrial DNA originating from tissue of a human having a disorder that is associated with late onset diabetes mellitus, contacting a predetermined quantity of the compound with the cybrid cells, measuring a mitochondrial complex V activity that is affected by mutant
20 mitochondrial DNA in the cybrid cells, and correlating a change in the mitochondrial complex V activity with effectiveness of the compound in the diagnosis or treatment of diabetes mellitus. Within certain embodiments, the ρ^0 cell line is immortal.

In another aspect, the invention provides a method for detecting the presence of diabetes mellitus in a human subject, by obtaining a biological sample
25 containing mitochondria from the subject and determining the presence of at least one polypeptide encoded by a mitochondrial ATP synthase gene. The presence of a polypeptide encoded by a mitochondrial ATP synthase gene may be determined using at least one monoclonal antibody or polyclonal antibody.

Within another aspect, a method for treating an individual afflicted with
30 diabetes mellitus is provided. The method generally comprises selectively introducing

into the mitochondria of the individual a conjugate molecule comprising a targeting molecule conjugated to a toxin, therapeutically useful agent or an imaging ligand via a linker, wherein the targeting molecule recognizes a mutant mitochondrial ATP synthase 8/6 or tRNA^{Lys} gene or its transcript or expressed product. Within certain embodiments, the targeting molecule may be an antibody, nucleic acid, protein or small molecule. The linker may contain a functional group selected from ester, ether, thioether, phosphorodiester, thiophosphorodiester, carbonate, carbamate, hydrazone, oxime, amino and amide groups. In certain embodiments, the imaging ligand may be a radioisotope, hapten, biotin, enzyme, fluorophore or chemilumiphore. Within preferred 10 embodiments, the toxin is phosphate, thiophosphate, dinitrophenol, maleimide or antisense oligonucleic acids. Within other embodiments, the therapeutically useful compound is an antioxidant or free radical trapping agent or other useful molecule. Examples of useful antioxidants according to the invention include but need not be limited to vitamin E and its analogs, hindered phenols, ascorbate derivatives, and 15 acetylcysteine and its derivatives, or other antioxidants known to those skilled in the art. Useful free radical trapping agents include but need not be limited to nitrones, for example phenyl-tert-butyl nitron derivatives and other free radical trapping agents known in the art.

The present invention offers outstanding opportunities to identify, probe 20 and characterize defective mitochondrial genes and mutations thereof that are associated with diabetes mellitus, to determine their cellular and metabolic phenotypes, and to assess the effects of various drugs and treatment regimens. In one embodiment, mitochondria from cells of a diabetes mellitus patient are transferred to immortalized ρ^0 cells that are β cells or insulin-responsive cells or other suitable cell types for 25 determination of defective mitochondrial gene structure and function. The cells undergo phenotypic changes characteristic of late onset diabetes mellitus; for example, reduced mitochondrial complex V activity that may be observed as ATP synthase activity, reduced hexokinase activity or increased production of free oxygen radicals. If exogenous agents or treatments are used on such samples and are able to prevent, delay,

or attenuate the phenotypic change, then those agents or treatments may be therapeutically beneficial.

Because such cell systems are observed to undergo changes in mitochondrial complex V activity characteristic of those observed in diabetes, they may also be used as methods of diagnosis. For example, cells are taken from an individual presenting with symptoms of late onset diabetes mellitus, and the mitochondria from those cells placed into immortalized β cells or insulin-responsive cells. Samples of these cultures are then chemically induced to differentiate into cells with pancreatic "beta cell-like" properties (*e.g.*, insulin secretion) or insulin responsiveness. If the differentiated cells that contain the patient's mitochondria begin to exhibit the degenerative phenotype that is characteristic of late onset diabetes mellitus (*e.g.*, impaired insulin secretion, decreased ATP synthesis, increased reactive oxygen species), this confirms that the mitochondria carry one or more causative mtDNA mutation(s). It thus confirms the diagnosis of late onset diabetes mellitus.

The invention also provides model systems for the screening of drugs and the evaluation of therapies effective in treating disorders associated with mitochondrial defects that segregate with late onset diabetes mellitus.

The present invention also comprises the transplantation of mitochondria into undifferentiated germ cells or embryonic cells, to yield organisms having mitochondria that have been wholly or partially derived from cells of a diseased organism.

These and other aspects of the invention will become more apparent by reference to the following detailed description of the invention and attached drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates a reaction scheme for the preparation of several acridine orange derivatives useful for the targeting, detection and selective destruction of defective mitochondria.

Figures 2-5 illustrate reaction schemes for the preparation of several JC-1 derivatives useful for the targeting, detection and selective destruction of defective mitochondria.

Figure 6 depicts production of reactive oxygen species (ROS) by
5 NIDDM and control cybrid cells.

Figure 7 depicts the increased mutational burden of the ATP synthase 8/6 genes in NIDDM.

DETAILED DESCRIPTION OF THE INVENTION

The ability to ascertain which individuals are predisposed to develop
10 IGT and diabetes mellitus is of enormous medical significance. The elucidation of the molecular events that underlie the progression from IGT to NIDDM is a quantum leap in the understanding of these conditions. A method for delaying, minimizing or preventing the onset of IGT or diabetes mellitus represents a major therapeutic advance.

The present invention represents an effective diagnostic assay of
15 mitochondrial defects associated with late onset diabetes which is reliable at or before the earliest manifestations of late onset diabetes symptoms. Moreover, the invention also allows the suppression of the undesired biological activity associated with mutations and thus affords a therapeutic treatment for late onset diabetes.

Genetic defects in the mitochondrial genes that encode components of
20 the electron transport chain are implicated in the switch from IGT to NIDDM. Perturbations of this protein complex predictably lead to an alteration in the production of adenosine triphosphate (ATP), the main source of energy for cellular biochemical reactions.

When mitochondrial intracellular ATP levels drop, glucose transport into
25 cells is impaired, metabolism of glucose is slowed and insulin secretion is decreased, all critical events in the switch from IGT to diabetes mellitus. Affected tissues are striated muscle (the major insulin-sensitive tissue) and pancreatic beta cells (insulin secreting cells). These target tissues contain non-dividing terminally differentiated cells that are susceptible to accumulation of mtDNA mutations. Achieving a threshold level of

mutations in mtDNA in pancreatic beta cells may precipitate a drop in insulin secretion, providing a molecular mechanism for the switch in disease phenotype from IGT to diabetes mellitus. In addition, a similar mechanism may precipitate a loss of insulin responsiveness in muscle.

5 Certain critical enzymes in the metabolism of glucose (hexokinases) and insulin secretion require ATP for proper function. Hexokinases and in particular glucokinase are bound to porin, a voltage dependent anion channel, located within the outer mitochondrial membrane. Porin, in turn, is apposed to the adenine nucleotide translocator of the inner mitochondrial membrane. Together these protein complexes
10 form a conduit for delivery of ATP from the inner mitochondrial matrix to hexokinases bound to the outer membrane and for return of ADP generated by catalytic activity of these kinases. The ATP used by mitochondrial bound hexokinases is derived primarily from the mitochondrial matrix and not the cytoplasm. Hexokinases require mitochondrial ATP for activation.

15 ATP synthase is an important component of the electron transport chain, the cellular energy generating system located in the mitochondria of eukaryotic cells. ATP synthase, also known as complex V, is composed of at least eight subunits. At least six of these subunits are encoded by nuclear genes; the remaining two subunits (6 and 8) are encoded by mitochondrial genes. The two mitochondrial DNA encoded ATP
20 synthase subunits are referred to herein collectively as ATP synthase 8/6, or individually as ATP synthase 8 and ATP synthase 6. Complex V activity, and the influence of defective ATP synthase 8/6 on complex V activity, can be measured by assays known to those skilled in the art. For example by way of illustration and not limitation, complex V-mediated enzymatic modification of suitable substrate molecules
25 under reaction conditions known in the art can be measured spectrophotometrically.

Without wishing to be held to any particular theory, it has been postulated that the destructive effects of mutations in the ATP synthase genes arise from the production of oxygen radicals and other chemically unstable molecules due to collapse of the proton gradient across the intramitochondrial membrane. The effects of

such free radicals are expected to be cumulative, especially in view of the lack of mechanisms for suppressing mutations in mitochondria.

It is important to an understanding of the present invention to note that all technical and scientific terms used herein, unless otherwise defined, are intended to have the same meaning as commonly understood by one of ordinary skill in the art. The techniques employed herein are also those that are known to one of ordinary skill in the art, unless stated otherwise.

The terms used herein are not intended to be limiting of the invention. For example, the term "gene" includes cDNAs, RNA, or other polynucleotides that encode gene products. In using the terms "nucleic acid", "RNA", "DNA", *etc.*, there is no intention to limit the chemical structures that can be used in particular steps. For example, it is well known to those skilled in the art that RNA can generally be substituted for DNA, and as such, the use of the term "DNA" should be read to include this substitution. In addition, it is known that a variety of nucleic acid analogues and derivatives can be made and will hybridize to one another and to DNA and RNA, and the use of such analogues and derivatives is also within the scope of the present invention. "Expression" of a gene or nucleic acid encompasses not only cellular gene expression, but also the transcription and translation of nucleic acid(s) in cloning systems and in any other context. "At least one mutation" denotes the substitution, addition or deletion of at least one nucleotide anywhere in the mitochondrial genome that is not present in a wild-type mitochondrial genome, whose phenotype correlates with diabetes mellitus. "Point mutations" are mutations within a nucleotide sequence that result in a change from one nucleotide to another; "silent mutations" are mutations that do not result in a change in the amino acid sequence encoded by the nucleotide sequence.

The term "tissue" includes blood and/or cells isolated or suspended from solid body mass, as well as the solid body mass of the various organs. "Immortal" cell lines denotes cell lines that are so denoted by persons of ordinary skill, or are capable of being passaged preferably an indefinite number of times, but not less than ten times, without significant phenotypical alteration. " p^0 cells" are cells essentially depleted of

functional mitochondria and/or mitochondrial DNA, by any method useful for this purpose.

The term "diabetes mellitus" is used in the claims to denote the disease that exhibits the symptoms of diabetes mellitus recognizable to one of ordinary skill in the art. "Diabetes mellitus" or "diabetes" may include, but need not be limited to, insulin-deficient non-insulin dependent diabetes mellitus (NIDDM) and insulin-dependent diabetes mellitus (IDDM). A phenotypic trait, symptom, mutation or condition "correlates" with diabetes mellitus if it is repeatedly observed in individuals diagnosed as having some form of diabetes mellitus, or if it is routinely used by persons of ordinary skill in the art as a diagnostic criterion in determining that an individual has diabetes mellitus or a related condition. Examples include: impaired insulin secretion, impaired response to insulin, or both.

Pre-clinical and/or asymptomatic conditions that correlate with the presence of mitochondrial mutations often observed in patients with diabetes mellitus, such as IGT, may represent steps in the progression in the disease. Individuals that lack the full panoply of such symptoms but carry mutations that "correlate" with diabetes mellitus are hereby defined as being "at risk" or having a "predisposition" for developing the fully symptomatic disease.

Although the invention focuses preferentially on humans afflicted with or at risk for developing diabetes mellitus as defined above, the invention also encompasses the analysis of tissues and preparations from relatives of persons having or being "at risk" for developing diabetes mellitus (which relatives may or may not themselves be at risk), and *in vivo* and *in vitro* animal and tissue culture models that may exhibit one or more or all of the symptoms that correlate with the mitochondrial mutations of the invention.

Reference to particular buffers, media, reagents, cells, culture conditions and the like, or to some subclass of same, is not intended to be limiting, but should be read to include all such related materials that one of ordinary skill in the art would recognize as being of interest or value in the particular context in which that discussion is presented. For example, it is often possible to substitute one buffer system or culture

medium for another, such that a different but known way is used to achieve the same goals as those to which the use of a suggested method, material or composition is directed.

The present invention provides cell lines whose genomic DNA is derived from cells that maintain a normal pancreatic β cell or insulin-responsive phenotype (such as, but not limited to, β TC6-F7, HIT, RINm5f, SH-SY5Y, TC-1 cells and INS-1 cells) and mitochondrial DNA having its origin in a human tissue sample derived from an individual with a disorder known to be associated with a mitochondrial defect that segregates with late onset diabetes mellitus. The present invention also provides an immortal ρ^0 cell line that is undifferentiated, but is capable of being induced to differentiate, comprising cultured immortal cells having genomic DNA with origins in immortalized β cells or insulin-responsive cells (for example, TC6-F7, HIT-T15, RINm5f, TC-1, and INS-1 cells), and mitochondrial DNA having its origin in a human tissue sample derived from an individual with a disorder known to be associated with a mitochondrial defect that segregates with late onset diabetes mellitus.

Although the cells suggested for certain embodiments herein are immortalized pancreatic β cells, adipocytes, neuronal tissue and cells, myoblasts and insulin-responsive cells and platelets, the present invention is not limited to the use of such cells. Cells from different tissues (breast epithelium, colon, lymphocytes, *etc.*) or different species (human, mouse, *etc.*) are also useful in the present invention.

Throughout this application various publications are referenced within parentheses. The disclosures of these publications in their entireties are hereby incorporated by reference in this application.

Diagnostic Detection of Late Onset Diabetes-Associated Mutations Using Hybridization and Ligation Techniques

In one aspect of the present invention, base changes in the mitochondrial ATP synthase or tRNA^{Lys} genes are detected and used as a diagnostic for late onset diabetes. A variety of techniques are available for isolating DNA and RNA from patient blood samples and for detecting mutations in isolated mitochondrial ATP

synthase and tRNA^{Lys} genes. For example, the DNA from a blood sample is obtained by cell lysis following alkali treatment. Often, there are multiple copies of RNA message per DNA. Accordingly, it is useful from the standpoint of detection sensitivity to have a sample preparation protocol which isolates both forms of nucleic acid. Total
5 nucleic acid may be isolated by guanidium isothiocyanate/phenol-chloroform extraction, or by proteinase K/phenol-chloroform treatment. Commercially available sample preparation methods such as those from Qiagen Inc. (Chatsworth, CA) are also utilized.

As discussed more fully hereinbelow, hybridization with one or more of
10 labelled probes containing the variant sequences under stringency conditions that result in specific binding to sequences complementary to these probes enables detection of late onset diabetes mutations. Since each late onset diabetes patient can be heteroplasmic (possessing both the late onset diabetes mutations and the normal sequence), a quantitative or semi-quantitative measure (depending on the detection
15 method) of such heteroplasmy is obtained by comparing the amount of signal from the late onset diabetes probe to the amount from the wild-type (normal) probe.

Certain techniques, discussed more fully hereinbelow, are available for detecting specific mutations in the mitochondrial ATP synthase and tRNA^{Lys} genes. The detection methods include, for example, cloning and sequencing, ligation of
20 oligonucleotides, use of the polymerase chain reaction and variations thereof (e.g., a PCR that uses 7-deaza-GTP), use of single nucleotide primer-guided extension assays, hybridization techniques using target-specific oligonucleotides that can be shown to preferentially bind to complementary sequences under given stringency conditions, and sandwich hybridization methods.

25 Cloning and sequencing of the ATP synthase and/or tRNA^{Lys} genes serves to detect late onset diabetes mutations in patients. Sequencing may be carried out with commercially available automated sequencers utilizing labelled primers or terminators. An alternate sequencing strategy is sequencing by hybridization using high density oligonucleotide arrays on silicon chips (Fodor et al., *Nature* 364:555-556
30 (1993); Pease et al., *Proc. Natl. Acad. Sci. USA* 91:5022-5026 (1994)). Labelled target

nucleic acid generated, for example, from PCR amplification of the target genes using fluorescently labelled primers, is hybridized with a chip containing a set of short oligonucleotides which probe regions of complementarity with the target sequence. The resulting hybridization patterns are used to reassemble the original target DNA sequence.

Mutational analysis is also carried out by ligation reaction methods based on ligation of oligonucleotide sequences which anneal immediately adjacent to each other on a target DNA or RNA molecule, including but not limited to the Ligase Chain Reaction or any other methods for the detection of specific mutations in nucleic acid sequences that are known to those skilled in the art (Wu and Wallace, *Genomics* 4:560-569 (1989); Landren et al., *Science* 241:1077-1080 (1988); Nickerson et al., *Proc. Natl. Acad. Sci.* 87:8923-8927 (1990); Barany, F., *Proc. Natl. Acad. Sci.* 88:189-193 (1991)). Ligase-mediated covalent attachment occurs only when the oligonucleotides are correctly base-paired. The Ligase Chain Reaction (LCR), which utilizes the thermostable *Taq* ligase for target amplification, is particularly useful for interrogating late onset diabetes mutation loci. The elevated reaction temperatures permits the ligation reaction to be conducted with high stringency (Barany, F., *PCR Methods and Applications* 1:5-16 (1991)).

Analysis of point mutations in DNA may also be carried out using polymerase chain reaction (PCR) and variations thereof (e.g., using 7-deaza GTP with or instead of dGTP). Mismatches are detected by competitive oligonucleotide priming under hybridization conditions where binding of the perfectly matched primer is favored (Gibbs et al., *Nucl. Acids Res.* 17:2437-2448 (1989)). In the amplification refractory mutation system technique (ARMS), primers are designed to have perfect matches or mismatches with target sequences either internal or at the 3' residue (Newton et al., *Nucl. Acids Res.* 17:2503-2516 (1989)). Under appropriate conditions, only the perfectly annealed oligonucleotide functions as a primer for the PCR reaction, thus providing a method of discrimination between normal and mutant (late onset diabetes) sequences.

Genotyping analysis of the ATP synthase and tRNA^{Lys} genes may also be carried out using single nucleotide primer-guided extension assays, where the specific incorporation of the correct base is provided by the high fidelity of the DNA polymerase (Syvanen et al., *Genomics* 8:684-692 (1990); Kuppuswamy et al., *Proc. Natl. Acad. Sci. USA* 88:1143-1147 (1991)). Another primer extension assay which allows for the quantification of heteroplasmy by simultaneously determining both wild-type and mutant nucleotides, is disclosed in United States Application Number 08/410,658 and United States Application Number 08/810,599, the disclosures of which are incorporated by reference.

Detection of single base mutations in target nucleic acids is conveniently accomplished by differential hybridization techniques using target-specific oligonucleotides (Suggs et al., *Proc. Natl. Acad. Sci.* 78:6613-6617 (1981); Conner et al., *Proc. Natl. Acad. Sci.* 80:278-282 (1983); Saiki et al., *Proc. Natl. Acad. Sci.* 86:6230-6234 (1989)). Mutations are diagnosed on the basis of the higher thermal stability of the perfectly matched probes as compared to the mismatched probes. The hybridization reactions are carried out in a filter-based format, in which the target nucleic acids are immobilized on nitrocellulose or nylon membranes and probed with oligonucleotide probes. Any of the known hybridization formats may be used, including Southern blots, slot blots, "reverse" dot blots, solution hybridization, solid support based sandwich hybridization, bead-based, silicon chip-based and microtiter well-based hybridization formats.

An alternative strategy involves detection of the ATP synthase and/or tRNA^{Lys} genes by sandwich hybridization methods. In this strategy, the mutant and wildtype (nominal) target nucleic acids are separated from non-homologous DNA/RNA using a common capture oligonucleotide immobilized on a solid support and detected by specific oligonucleotide probes tagged with reporter labels. The captured oligonucleotides are immobilized on microtitre plate wells or on beads (Gingeras et al., *J. Infect. Dis.* 164:1066-1074 (1991); Richman et al., *Proc. Natl. Acad. Sci.* 88:11241-11245 (1991)).

While radio-isotopic labeled detection oligonucleotide probes are highly sensitive, non-isotopic labels are preferred due to concerns about handling and disposal of radioactivity. A number of strategies are available for detecting target nucleic acids by non-isotopic means (Matthews et al., *Anal. Biochem.* 169:1-25 (1988)). The non-isotopic detection method may be either direct or indirect.

In an indirect detection process, the oligonucleotide probe is generally covalently labelled with a hapten or ligand such as digoxigenin (DIG) or biotin. Following the hybridization step, the target-probe duplex may be detected by an antibody- or streptavidin-enzyme complex. Enzymes commonly used in DNA diagnostics are horseradish peroxidase and alkaline phosphatase. One particular indirect method, the Genius detection system (Boehringer Mannheim) may be especially useful for mutational analysis of mitochondrial genes. This indirect method uses digoxigenin as the tag for the oligonucleotide probe and is detected by an anti-digoxigenin-antibody-alkaline phosphatase conjugate.

Direct detection methods include the use of fluorophor-labeled oligonucleotides, lanthanide chelate-labeled oligonucleotides or oligonucleotide-enzyme conjugates. Examples of fluorophor labels are fluorescein, rhodamine and phthalocyanine dyes. Examples of lanthanide chelates include complexes of Eu^{3+} and Tb^{3+} . Directly labeled oligonucleotide-enzyme conjugates are preferred for detecting point mutations when using target-specific oligonucleotides, as they provide very high sensitivities of detection.

Oligonucleotide-enzyme conjugates are prepared by a number of methods (Jablonski et al., *Nucl. Acids Res.* 14:6115-6128 (1986); Li et al., *Nucl. Acids Res.* 15:5275-5287 (1987); Ghosh et al., *Bioconjugate Chem.* 1:71-76 (1990)), with alkaline phosphatase being the enzyme of choice for obtaining high sensitivities of detection. The detection of target nucleic acids using these conjugates may be carried out by filter hybridization methods or by bead-based sandwich hybridization (Ishii et al., *Bioconjugate Chemistry* 4:34-41 (1993)).

Detection of the probe label may be accomplished using the following approaches. For radioisotopes, detection may be by autoradiography, scintillation

counting or phosphor imaging. For hapten or biotin labels, probe may be detected by antibody or streptavidin bound to a reporter enzyme such as horseradish peroxidase or alkaline phosphatase, which is then detected by enzymatic means. For fluorophor or lanthanide-chelate labels, fluorescent signals may be measured with spectrofluorimeters with or without time-resolved mode or using automated microtitre plate readers. With enzyme labels, detection may be by color or dye deposition (p-nitrophenyl phosphate or 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium for alkaline phosphatase and 3,3'-diaminobenzidine-NiCl₂ for horseradish peroxidase), fluorescence (e.g., 4-methyl umbelliferyl phosphate for alkaline phosphatase) or chemiluminescence (the alkaline phosphatase dioxetane substrates LumiPhos 530 from Lumigen Inc., Detroit MI or AMPPD and CSPD from Tropix, Inc.). Chemiluminescent detection may be carried out with X-ray or polaroid film or by using single photon counting luminometers. This is the preferred detection format for alkaline phosphatase labelled probes.

The detection oligonucleotide probes range in size between 10-100 bases, and are preferably between 15 to 30 bases in length. In order to obtain the required target discrimination using the detection oligonucleotide probes, the hybridization reactions are generally run between 20°-60°C, and most preferably between 30°-50°C. As known to those skilled in the art, optimal discrimination between perfect and mismatched duplexes is obtained by manipulating the temperature and/or salt concentrations or inclusion of formamide in the stringency washes.

Diagnostic Detection of Diabetes Associated Mutations Using Antibodies

As an alternative to detection of mutations in the nucleic acids associated with the mutant mitochondrial genes described herein, the protein products of these genes may be analyzed using immune techniques. In particular, altered proteins (variant polypeptides) encoded by nucleic acids having point mutations in ATP synthase subunit 8 may be isolated and used to prepare antisera and monoclonal antibodies that specifically detect the products of the mutated genes, and not those of non-mutated or wild-type genes. Mutated gene products may also be used to immunize animals for the production of polyclonal antibodies. Recombinantly produced peptides

can also be used to generate polyclonal antibodies. These peptides represent small fragments of gene products produced by expressing regions of the mitochondrial genome containing point mutations.

Antibodies provided by the invention may be polyclonal antibodies or
5 monoclonal antibodies. Antibodies may also be fragments of monoclonal or polyclonal antibodies that retain antibody binding activities. "Antibodies" further includes recombinant or genetically engineered antibodies, or fragments thereof or fusion proteins having antibody activity, including single-chain antibodies.

As discussed, for example, in PCT/US93/10072, variant polypeptides
10 encoded by nucleic acids with point mutations in ATP synthase subunit 8 are used to immunize an animal for the production of polyclonal antiserum. For example, a recombinantly produced fragment of a variant polypeptide is injected into a mouse along with an adjuvant so as to generate an immune response. Murine immunoglobulins which bind the recombinant fragment with a binding affinity of at
15 least $1 \times 10^7 \text{ M}^{-1}$ are harvested from the immunized mouse as an antiserum, and are optionally further purified by affinity chromatography or other means. Additionally, spleen cells are harvested from the mouse and fused to myeloma cells to produce a bank of antibody-secreting hybridoma cells. The bank of hybridomas is screened for clones that secrete immunoglobulins which bind the recombinantly produced fragment with an
20 affinity of at least $1 \times 10^6 \text{ M}^{-1}$. More specifically, immunoglobulins that selectively bind to the variant polypeptides but poorly or not at all to wild-type polypeptides are selected, either by pre-absorption with wild-type proteins or by screening of hybridoma cell lines for specific idiotypes that bind the variant, but not wild-type, polypeptides.

Nucleic acid sequences capable of ultimately expressing the desired
25 variant polypeptides are formed from a variety of different polynucleotides (genomic or cDNA, RNA, synthetic oligonucleotides, *etc.*) as well as by a variety of different techniques.

The DNA sequences are expressed in hosts after the sequences have been operably linked to (*i.e.*, positioned to ensure the functioning of) an expression control
30 sequence. These expression vectors are typically replicable in the host organisms either

as episomes or as an integral part of the host chromosomal DNA. Commonly, expression vectors contain selection markers (e.g., markers based on tetracycline resistance or hygromycin resistance) to permit detection and/or selection of those cells transformed with the desired DNA sequences. Further details can be found in U.S. Patent No. 4,704,362, incorporated herein by reference thereto.

Polynucleotides encoding a variant polypeptide include sequences that facilitate transcription (expression sequences) and translation of the coding sequences such that the encoded polypeptide product is produced. Construction of such polynucleotides is well known in the art. For example, such polynucleotides include a promoter, a transcription termination site (polyadenylation site in eukaryotic expression hosts), a ribosome binding site, and, optionally, an enhancer for use in eukaryotic expression hosts, and, optionally, sequences necessary for replication of a vector.

E. coli is one prokaryotic host useful particularly for cloning DNA sequences of the present invention. Other microbial hosts suitable for use include bacilli, such as *Bacillus subtilis*, and other enterobacteriaceae, such as *Salmonella*, *Serratia*, and various *Pseudomonas* species. Expression vectors made in these prokaryotic hosts will typically contain expression control sequences compatible with the host cell (e.g., an origin of replication). In addition, any number of a variety of well-known promoters may be used, such as the lactose promoter system, a tryptophan (Trp) promoter system, a beta-lactamase promoter system, or a promoter system from phage lambda. The promoters typically control expression, optionally with an operator sequence, and have ribosome binding site sequences, for example, for initiating and completing transcription and translation.

Other microbes, such as yeast, may also be used for expression. *Saccharomyces* is a suitable host with suitable vectors having expression control sequences, such as promoters, including 3-phosphoglycerate kinase or other glycolytic enzymes, and an origin of replication, termination sequences, etc., as desired.

In addition to microorganisms, mammalian tissue cell culture may be used to express and produce the polypeptides of the present invention. Eukaryotic cells are preferred, because a number of suitable host cell lines capable of secreting intact

human proteins have been developed in the art, including CHO cell lines, various COS cell lines, HeLa cells, myeloma cell lines, Jurkat cells, and so forth. Expression vectors for these cells include expression control sequences, such as an origin of replication, a promoter, an enhancer, and necessary information processing sites, such as ribosome binding sites, RNA splice sites, polyadenylation sites, and transcriptional terminator sequences. Preferred expression control sequences include promoters derived from immunoglobulin genes, SV40, Adenovirus, Bovine Papilloma Virus, and so forth. The vectors containing the DNA segments of interest (*e.g.*, sequences encoding a variant polypeptide) are transferred into the host cell by well-known methods, which vary depending on the type of cellular host. For example, calcium chloride transfection is commonly utilized for prokaryotic cells, whereas calcium phosphate treatment or electroporation is useful for other cellular hosts.

Test Kits

The methods described herein readily lend themselves to the formulation of test kits for use in diagnosis. Such a kit comprises a carrier compartmentalized to receive in close confinement one or more containers, wherein a first container contains suitably labeled DNA or immunological probes. Other containers generally contain reagents useful in the localization of the labeled probes, such as enzyme substrates. Still other containers contain restriction enzymes, buffers, *etc.*, together with instructions for use.

Antisense Embodiments

Protein synthesis may be inhibited through the use of antisense or triplex oligonucleotides, analogues or expression constructs. These methods entail introducing into a cell a nucleic acid sufficiently complementary in sequence so as to specifically hybridize to a target nucleic acid. Antisense methodology inhibits the normal processing, translation or half-life of the target nucleic acid. A variety of antisense methods are well known to one skilled in the art. (Hélenè et al., *Biochem. Biophys. Acta* 1049:99-125 (1990).) Procedures for inhibiting gene expression in cell culture and

in vivo are described, for example, by C.F. Bennett et al. (*J. Liposome Res.* 3:85 (1993) and C. Wahlestedt et al., *Nature* 363:260 (1993)).

Suppressing the effects of the mutations through antisense technology provides an effective therapy for diabetes mellitus. Antisense agents target
5 mitochondrial DNA, by triplex formation with double-stranded DNA, by duplex formation with single stranded DNA during transcription, or both. Antisense agents also target messenger RNA coding for mutated ATP synthase gene(s). Since the sequences of both the DNA and the mRNA are essentially the same, it is not necessary to determine accurately the precise target to account for the desired effect.

10 As used herein, an "antisense" oligonucleotide is one that base pairs with single stranded DNA or RNA by Watson-Crick base pairing and with duplex target DNA via Hoogsteen hydrogen bonds. Antisense and triplex methods generally involve the treatment of cells or tissues with a relatively short oligonucleotide, although longer sequences may be used to achieve inhibition. The oligonucleotide is either deoxyribo-
15 or ribonucleic acid or analogues thereof, and must be of sufficient length to form a stable duplex or triplex with the target RNA or DNA at physiological temperatures and salt concentrations. It should also be of sufficient complementarity or sequence specificity to specifically hybridize to the target nucleic acid. Oligonucleotide lengths sufficient to achieve this specificity are generally about 10 to 60 nucleotides long,
20 preferably about 10 to 20 nucleotides long. However, hybridization specificity is not only influenced by length and physiological conditions but may also be influenced by such factors as GC content and the primary sequence of the oligonucleotide. Such principles are well known in the art.

The composition of the antisense or triplex oligonucleotides influences
25 the efficiency of inhibition. For example, it is preferable to use oligonucleotides that are resistant to degradation by the action of endogenous nucleases. Nuclease resistance will confer a longer *in vivo* half-life to the oligonucleotide, thus increasing its efficacy and reducing the required dose.

Antisense therapy is extremely efficient since only a few copies per cell
30 are required to achieve complete inhibition. Greater efficacy is obtained by modifying

the oligonucleotide so that it is more permeable to cell membranes. Such modifications are well known in the art and include the alteration of the negatively charged phosphate backbone bases, or modification of the sequences at the 5' or 3' terminus with agents such as intercalators and cross-linking molecules. Specific examples of such
5 modifications include oligonucleotide analogs that contain methylphosphonate (Miller, P.S., *Biotechnology* 2:358-362 (1991)), phosphorothioate (Stein, *Science* 261:1004-1011 (1993)) and phosphorodithioate linkages (Brill, W. K-D., *J. Am. Chem. Soc.* 111:2322 (1989)). Other types of linkages and modifications exist as well, such as a polyamide backbone in peptide nucleic acids (Nielson et al., *Science* 254:1497
10 (1991)), formacetal (Matteucci, M., *Tetrahedron Lett.* 31:2385-2388 (1990)) carbamate and morpholine linkages as well as others known to those skilled in the art.

Vectors containing antisense nucleic acids may be employed to express antisense message to reduce the expression of the target nucleic acid, and therefore its activity. Such vectors are known or can be constructed by those skilled in the art and
15 should contain all expression elements necessary to achieve the desired transcription of the antisense or triplex sequences. Other beneficial characteristics may also be contained within the vectors, such as mechanisms for recovery of the nucleic acids in a different form.

Phagemids are a specific example of such beneficial vectors because
20 they are used either as plasmids or as bacteriophage vectors. Examples of other vectors include viruses, such as bacteriophages, baculoviruses and retroviruses, cosmids, plasmids, liposomes and other recombination vectors. The vectors may also contain elements for use in either procaryotic or eukaryotic host systems. One of ordinary skill in the art will know which host systems are compatible with a particular vector.

25 The vectors may be introduced into cells or tissues by any one of a variety of known methods within the art. Such methods are described for example in Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, New York (1992), which is hereby incorporated by references, and in Ausubel et al., *Current Protocols in Molecular Biology*, John Wiley and Sons,
30 Baltimore, MD (1989), which is also hereby incorporated by reference. The methods

include, for example, stable or transient transfection, lipofection, electroporation and infection with recombinant viral vectors.

Introduction of nucleic acids by infection offers several advantages. Higher efficiency is achieved due to their infectious nature. Moreover, viruses are very specialized and typically infect and propagate in specific cell types. Thus, their natural specificity is used to target the antisense vectors to specific cell types *in vivo* or within a tissue or mixed culture of cells. Viral vectors may also be modified with specific receptors or ligands to alter target specificity through receptor mediated events.

A specific example of a viral vector for introducing and expressing antisense nucleic acids is the adenovirus derived vector Adenop53TX. This vector expresses a herpes virus thymidine kinase (TK) gene for either positive or negative selection and an expression cassette for desired recombinant sequences such as antisense sequences. This vector may be used to infect cells including most cancers of epithelial origin, glial cells and other cell types. This vector, as well as others that exhibit similar desired functions, may be used to treat a mixed population of cells to selectively express the antisense sequence of interest. A mixed population of cells can include, for example, *in vitro* or *ex vivo* culture of cells, a tissue or a human subject.

Additional features can be added to the vector to ensure its safety and/or enhance its therapeutic efficacy. Such features include, for example, markers that are used to negatively select against cells infected with the recombinant virus. An example of such a negative selection marker is the TK gene, which confers sensitivity to the antibiotic gancyclovir. Negative selection is therefore a means by which infection is controlled because it provides inducible suicide through the addition of antibiotics. Such protection ensures that if, for example, mutations arise that produce mutant forms of the viral vector or antisense sequence, cellular transformation will not occur. Moreover, features that limit expression to particular cell types can also be included. Such features include, for example, promoter and expression elements that are specific for the desired cell type.

In addition to the specificity afforded by the antisense agents, the target RNA or genes may be irreversibly modified by incorporating reactive functional groups in these molecules which covalently link the target sequences, *e.g.*, by alkylation.

In a preferred embodiment, antisense agents target messenger RNA
5 coding for the mutated ATP synthase or tRNA^{Lys}. Since the sequences of both the DNA and the mRNA are the same, it is not necessary to determine accurately the precise target to account for the desired effect.

To demonstrate the ability to affect expression of mitochondrial ATP synthase or tRNA^{Lys} genes, an oligonucleotide designed to hybridize near the 5'-end of
10 the wild type ATP synthase or tRNA^{Lys} gene or gene transcript is synthesized. When the antisense oligonucleotide is present in a suitable cell culture, the cells will die if the electron transport chain is interrupted. Control fibroblasts treated with complementary ('sense') oligonucleotide, or left untreated, will exhibit no such effects.

Since the diagnostic test of the present invention may be used to
15 determine which of the specific late onset diabetes mutations exists in a particular late onset diabetes patient, one can "customize" treatment of the patient with antisense oligonucleotides directed only to the detected mutations. When combined with the present diagnostic test, this approach to "patient-specific therapy" results in treatment restricted to the specific mutations detected in a patient.

20 Antisense oligonucleotide therapeutic agents with a high degree of pharmaceutical specificity allow for the combination of two or more antisense therapeutics at the same time, without increased cytotoxic effects. Thus, when a patient is diagnosed as having two or more late onset diabetes mutations in ATP synthase or tRNA^{Lys} genes, the therapy may be tailored to treat the multiple mutations
25 simultaneously. This patient-specific therapy circumvents the need for 'broad spectrum' antisense treatment using all possible mutations and minimizes the exposure of the patient to any unnecessary antisense therapeutic treatment. The end result is less costly treatment, with less chance for toxic side effects.

Depletion of Mitochondria

The present invention also provides methods for the selective destruction of target mitochondria and the accumulation of therapeutically useful agents into target mitochondria. Since the mitochondrial genome is heteroplasmic (*i.e.*, it contains mutated and normal DNA), destruction of target mitochondria carrying mutations will leave intact mitochondria carrying normal or wild-type DNA and these normal mitochondria will repopulate the targeted tissue, thereby normalizing mitochondrial function. Alternatively, accumulating a therapeutically useful agent into mutant or wild-type DNA carrying mitochondrial mutations will protect these mitochondria from further damage.

This selective destruction or drug targeting can be accomplished by identifying unique characteristics of mitochondria carrying mutated DNA, designing a small molecule that is directed at one or more of these unique characteristics, and conjugating a mitochondrial toxin or therapeutically useful agent to this small molecule. (A "targeting molecule" is any molecule that selectively accumulates in mitochondria having defective ATP synthase or tRNA^{Lys}, and includes antibodies, nucleic acids, proteins, and small molecules that recognize a mutant mitochondrial ATP synthase 8/6 or tRNA^{Lys} gene or its transcript or expressed product. "Mitochondrial toxins" are molecules that destroy or disable the selected mitochondria, and include phosphate, thiophosphate, dinitrophenolate, maleimide and antisense oligonucleotides such as those discussed above.) The toxin will be concentrated within the defective mitochondria by the targeting molecule and will selectively disable or destroy the defective mitochondria. Therapeutically useful compounds are molecules that interfere with the production of oxygen radicals or trap oxygen radicals in an inert form once they are produced, such as, for example, antioxidants and radical spin trapping agents.

The molecule may be an active mitochondrial toxin or therapeutic agent in its conjugated form. However, it is preferred to design the molecule such that it is inactive in its conjugated form.

The chemical linkage between the targeting molecule and the toxin or therapeutic agent may be a substrate for a mitochondria-specific enzyme or be sensitive

to redox cleavage. Choice of the linkage depends upon the chemical nature of the targeting molecule and toxin and the requirements of the cleavage process. Once the conjugate is concentrated in the defective mitochondria, the toxin or therapeutic agent is cleaved from the targeting molecule, activating the toxin or releasing the therapeutic agent.

Mitochondria with defective ATP synthase activity exhibit decreased synthesis of adenosine triphosphate and general bioenergetic failure. As a consequence, mitochondria carrying mutated DNA will become enlarged and the intra-mitochondrial membrane potential will increase.

Enlarged mitochondria have increased levels of cardiolipin and other negatively charged phospholipids. The acridine orange derivative 10N-nonylacridine orange (NAO) binds relatively specifically to cardiolipin and accumulates in dysfunctional mitochondria. The accumulation of NAO and other chemical derivatives of acridine orange, including but not limited to those with aliphatic chains of variable length attached to the ring nitrogen of acridine orange ([3,6-bis (dimethyl-amino) acridine]), such as 10N-pentylacridine orange, 10N-octylacridine orange, and dodecylacridine orange, is independent of the mitochondrial transmembrane potential. (Maftah et al., *Biochemical and Biophysical Research Communications* 164(1):185-190 (1989)). At concentrations up to 1 μ M, NAO and its derivatives can be used to target other molecules to the inner mitochondrial matrix. If the NAO is chemically linked to a mitochondrial toxin such as phosphate, thiophosphate, dinitrophenol, maleimide and antisense oligonucleotides, or to a therapeutically useful molecule such as an antioxidant, radical spin trapping agent, etc., then mitochondria accumulating the NAO-mitochondrial toxin conjugate or therapeutic conjugate can be selectively disabled, destroyed or protected. Alternately, at high concentrations (3-10 μ M) NAO and its derivatives inhibit electron transport, ATP hydrolysis and P_i transport and disrupt respiration. (Maftah et al., *FEBS Letters* 260(2):236-240 (1990)). At these concentrations, NAO is mitochondrial toxin.

According to an embodiment of the present invention, the terminus of any aliphatic or other type of chain (such as polyethylene glycol) attached to the ring

nitrogen of acridine orange may be chemically derivatized with carboxylic acid, hydroxyl, sulfhydryl, amino or similar groups to accept any mitochondrial toxin. In other embodiments, additional sites of attachment of the mitochondrial toxin to acridine orange and acridine orange derivatives are selected. For example, the
5 10-N-(10-hydroxy-1-decyl)-3,6-bis(dimethylamino)acridine bromide salt may be prepared and further derivatized to 10-N-(10-phosphoryl-1-decyl)-3,6-bis(dimethylamino) acridine chloride salt or 10-N-(10-thiophosphoryl-1-decyl)-3,6-bis(dimethylamino)acridine chloride salt. Alternately, 10-N-(11-undecanoic acid)-3,6-bis(dimethylamino)acridine bromide salt
10 may be prepared and further derivatized to 10-N(11-undecan-1-oic acid 2,4-dinitrophenolate)-3,6-bis(dimethylamino) acridine bromide salt.

Upon cleavage, the phosphate, thiophosphate or dinitrophenol levels selectively increase within defective mitochondria and destroy them. The functionalization and covalent attachment of the toxin does not need to depend on
15 subsequent release of the toxin by cleavage of the NAO from the toxin, if the attachment point on the toxin is non-interfering with the function of the toxin within the mitochondria.

Several examples of the preparation of acridine orange derivatives are summarized in Figure 1 and in the examples below. Other modifications are permitted
20 as known to those skilled in the art.

Still other embodiments of the present invention target changes in the intramitochondrial membrane potential due to defective ATP synthase activity. Delocalized lipophilic cations have been used to monitor mitochondrial membrane potential. The uptake of these cations is related to the presence of the negative sink
25 inside the mitochondria created by the proton pump. As mitochondria increase in size due to ATP synthase or tRNA^{Lys} defects, the transmembrane potential will increase and these defective mitochondria will accumulate lipophilic cations. According to an embodiment of the present invention, these lipophilic cations are conjugated to mitochondrial toxins or therapeutically useful agents and used to destroy or protect
30 defective mitochondria that possess increased transmembrane potentials.

Rhodamine-123 has been used extensively to monitor mitochondrial membrane potential and can conjugate to mitochondrial toxins to concentrate toxins within the mitochondria. The compound 5, 5', 6, 6'-tetrachloro-1, 1', 3, 3'-tetraethylbenzimidazolo-carbocyanine iodide (JC-1) also accumulates in mitochondria dependent upon the transmembrane potential. When JC-1 exceeds a critical concentration, J-aggregates form in the mitochondrial matrix, and their size causes these JC-1 J-aggregates to diffuse slowly out of the mitochondria (Reers et al., *Biochemistry* 30(18):4480-4486 (1991)). JC-1 may be chemically conjugated to a mitochondrial toxin or therapeutically useful agent, producing a long-lived toxic or therapeutic compound to mitochondria displaying increased transmembrane potential relative to normal mitochondria.

As with NAO, by adding a functional group to the JC-1 structure one can covalently attach another chemical entity to the JC-1 subunit. Delivery to the cells then causes the dual agent to be preferentially transported into the mitochondria, where the dual agent may be cleaved at the covalent attachment to release a toxin or therapeutically useful agent within the mitochondria where it exerts the desired effect. Alternatively, the functionalization and covalent attachment of the toxin or therapeutically useful agent does not need to depend on subsequent release of the toxin or therapeutically useful agent by cleavage of the JC-1 from the active agent, if the attachment point on the active species is non-interfering with the function of the toxin or therapeutically useful agent within the mitochondria.

Figures 2, 3 and 4 outline the functionalization of JC-1 by several different methods. The examples hereinbelow illustrate an oxygen functionality, but the same can be accomplished with a nitrogen, sulfur or carboxylic acid functionality.

By utilizing the quasi-symmetrical nature of JC-1, a new chemical entity may be synthesized that is "half" JC-1 and contains a functional group capable of being used as a point for covalent attachment of another chemical entity to the JC-1 subunit. The existence of the JC-1 subunit facilitates selective transport of the whole molecule to the mitochondria where, if desired, enzymes effect cleavage of the JC-1 subunit from the toxin or therapeutically useful agent, allowing it to exert the desired effect.

Alternatively, the functionalization and covalent attachment of the toxin or therapeutically useful agent does not need to depend on subsequent release of the toxin or therapeutically useful agent by cleavage of the JC-1 subunit from the toxin or therapeutically useful agent, if the attachment point on the toxin or therapeutically useful agent is non-interfering with the function of the active agent within the mitochondria.

Figure 5 outlines the synthesis of a functionalized "half" JC-1 subunit by several different methods. The attachment of the active chemical species is via the heteroatom incorporated in the JC-1 or "half" JC-1 structure. This attachment may be accomplished by any number of linking strategies such as by taking advantage of a functionality on the active molecule (such as a carboxylic acid to form an ester with the oxygen of the altered JC-1) or by using a linker to space between the JC-1 and the toxin or therapeutically useful agent. These strategies are well known to those skilled in the chemistry of preparing diagnostic or labelling molecules with reporter functions for biological studies and include ester, amide, urethane, urea, sulfonamide, and sulfonate ester (S.T. Smiley et al., *Proc. Nat'l. Acad. Sci. USA* 88:3671-3675 (1991)).

As noted hereinabove, mitochondria carrying mutated ATP synthase or tRNA^{Lys} genes have increased levels of cardiolipin and other negatively charged phospholipids as well as increased mitochondrial membrane potential. As a result, the mitochondria selectively accumulate targeting molecules, including acridine orange derivatives and lipophilic cations such as rhodamine-123 and JC-1 derivatives. In addition to selectively introducing toxins or therapeutically useful agents into the mitochondria, such targeting molecules can also selectively introduce imaging ligands, which can form the basis of effective *in vivo* and *in vitro* diagnostic strategies. Such strategies include magnetic resonance imaging (MRI), single photon emission computed topography (SPECT), and positron emission tomography (PET). Preferred imaging ligands for the practice of the present invention include radioisotopes (such as ¹²³I, ¹²⁵I, ¹⁸F, ¹³N, ¹⁵O, ¹¹C, ^{99m}Tc, ⁶⁷Ga and so forth), haptens (such as digoxigenin), biotin, enzymes (such as alkaline phosphatase or horseradish peroxidase), fluorophores

(such as fluorescein lanthanide chelates, or Texas Red®), and gadolinium chelates for MRI applications. (Saha et al., *Seminars in Nuclear Medicine* 4:324-349 (1994).)

As an example of an *in vitro* diagnosis, a targeting molecule, such as an acridine orange or JC-1 derivative, is labelled with fluorescein as an imaging ligand.

- 5 The labelled targeting molecule is introduced into a human tissue cell culture such as a primary fibroblast culture. After a period of several hours, cells having mitochondria with defective ATP synthase or tRNA^{Lys} genes selectively absorb the labelled targeting molecule in amounts greater than cells without such mitochondria. The cells are then washed and sorted in a fluorescence activated cell sorter (FACS) such as that sold by
- 10 Becton Dickinson. Threshold limits can be established for the FACS using cells with wild-type mitochondria. Similarly, in an *in vivo* diagnosis, a targeting molecule such as an acridine orange or JC-1 derivative is labelled with ^{99m}Tc, ¹⁸F or ¹²³I as an imaging ligand. This labelled targeting molecule is introduced into the bloodstream of a patient. After a period of several hours, the labelled targeting molecule accumulates in those
- 15 tissues having mitochondria with mutated ATP synthase or tRNA^{Lys} genes. Such tissues can be directly imaged using positron-sensitive imaging equipment.

- Selective destruction of defective mitochondria may also be achieved by using ribozymes. Ribozymes are a class of RNA molecules that catalyze strand scission of RNA molecules independent of cellular proteins. Specifically, ribozymes may be
- 20 directed to hybridize and cleave target mitochondrial mRNA molecules. The cleaved target RNA cannot be translated, thereby preventing synthesis of essential proteins which are critical for mitochondrial function. The therapeutic application thus involves designing a ribozyme which incorporates the catalytic center nucleotides necessary for function and targeting it to mRNA molecules which encode for dysfunctional ATP
- 25 synthase subunits or tRNA^{Lys}. The ribozymes may be chemically synthesized and delivered to cells or they can be expressed from an expression vector following either permanent or transient transfection. Therapy is thus provided by the selective removal of mutant mRNAs in defective mitochondria.

Cybrids

Methods for depleting mitochondrial DNA ("mtDNA") from cells and then transforming those cells with mitochondria from other cells have been reported in the literature. (King, M.P. and Attardi, B., *Science* 246:500-503 (1989).) The value of the reported cell lines is limited because the pathogenesis of a given disease may depend on cell type. Also, the techniques for mitochondrial transformation of human cells allow only limited short term studies. Care has to be taken in growing cultures since transformed, undifferentiated cells containing wild-type mtDNA are healthier than those containing mutant mtDNA and therefore have a propagative advantage in culture. Over the course of several generations, cells with wild-type mtDNA would dominate the cellular population (*i.e.*, mutant mtDNA would be selected against) and cells containing mutated mtDNA would be lost.

The different embodiments of the present invention overcome these limitations. First, using ρ^0 cells derived from cultures of cell lines in which different cellular manifestations of diabetes mellitus are observed permits analysis of changes in the mitochondrial genome and closely mimics the functional effects of mitochondrial dysfunction in pancreatic β cells and insulin-responsive cells (*e.g.*, muscle, neurons, adipocytes, *etc.*). Secondly, by introducing mitochondria from diseased cells into an undifferentiated, immortal cell line, it is possible to maintain the transformants in culture almost indefinitely. Although it would be possible to study and use the undifferentiated cells themselves, it is preferred to take a sample of such cells, and then induce them to differentiate into the cell type that they are destined to become.

Mitochondria to be transferred to construct model systems in accordance with the present invention may be isolated from virtually any tissue or cell source. Cell cultures of all types-could potentially be used, as could cells from any tissue. However, cells that are implicated in insulin secretion or that are responsive to insulin, especially isolated pancreatic β cells, fibroblasts, brain tissue, myoblasts, and cell lines derived therefrom are preferred sources of donor mitochondria. Platelets are the most preferred, in part because of their ready abundance, and their lack of nuclear DNA. This

preference is not meant to constitute a limitation on the range of cell types that are used as donor sources.

Recipient cells useful to construct models in accordance with the present invention are potentially cells of any type that may be maintained in culture, but
5 immortalized cell lines are preferred because of their growth characteristics. Many such cell lines are commercially available, and new ones are isolated and rendered immortal by methods that are well known in the art. Although cultured cell lines are preferred, it is also possible that cells from another individual. *e.g.*, an unaffected close blood relative, are useful; this could have certain advantages in ruling out non-mitochondrial
10 effects. In any event, it is preferable to use recipient cells that can be induced to differentiate by the addition of particular chemical (*e.g.*, hormones, growth factors, *etc.*) or physical (*e.g.*, temperature, exposure to radiation such as U.V. radiation, *etc.*) induction signals.

It is most preferred that the recipient cells be selected such that they are
15 of (or capable of being induced to become) the type that is most phenotypically affected in diseased individuals. For example, for constructing models for mitochondrial defects associated with diabetes, immortalized pancreatic β cell lines are most preferred.

However, the present invention also contemplates that the recipient cell line is a member of the group of cell lines consisting of a mammalian zygote, an
20 embryonic cell capable of differentiating and giving rise to a tissue, an individual, or a germ cell line.

In some embodiments of the present invention mitochondria are transplanted into an immortal, differentiable cell line, and the transplanted cells are also immortal. The invention further teaches the induction of differentiation among a
25 subpopulation of the immortal culture, which allows for the same experiments to be done as would otherwise have been possible had the transplant been made directly into the differentiated cells. For example, mitochondria from an NIDDM or diabetes mellitus patient are transplanted into an immortalized pancreatic β cell or adipocyte or myoblast or a cell line derived therefrom, subcultures of which are induced to
30 differentiate into pancreatic β cells or fat or muscle cells. The phenotypic expression of

the mitochondrial defects in this model system is thus observed in the very cell type that is most affected by the disease.

The only requirement for the method of isolating mitochondria is that the mitochondria be substantially purified from the source cells and that the source cells be sufficiently disrupted that there is little likelihood that the source cells will grow and proliferate in the culture vessels to which the mitochondria are added for transformation. Mitochondrial DNA (mtDNA) of the target cells may be removed, for example, by treatment with ethidium bromide. Presumably, this works by interfering with transcription or replication of the mitochondrial genome, and/or by interfering with mRNA translation. The mitochondria are thus rendered unable to replicate and/or produce proteins required for electron transport, and the mitochondria shut down, apparently permanently. However, it is important to note that it is not necessary for the purposes of this invention to use any particular method to remove the mitochondria or mitochondrial DNA.

The cybrid cells of this invention are useful for evaluating chemical compounds for potential utility in the diagnosis or treatment of diabetes mellitus, which encompasses: reducing or delaying the risk of developing diabetes mellitus, and/or treatment of a symptom of diabetes mellitus or a condition that is associated with late onset diabetes mellitus, and/or establishing whether and to what extent a test compound is capable of causing a specified trait to become more similar to those of control cells having mitochondria that lack said defect. This is accomplished by a.) contacting a predetermined quantity of a test compound with cultured cybrid cells having genomic DNA originating from a p^0 cell line and mitochondrial DNA originating from tissue of a human having at least one mutation in an ATP synthase gene or a tRNA^{Lys} gene that is associated with late onset diabetes mellitus; and b.) measuring a phenotypic trait in said cybrid cells that is affected by said mitochondrial defect; and c.) establishing whether and to what extent said drug is capable of causing said trait to become more similar to those of control cells having mitochondria that lack said defect, which capability indicates that the compound has utility in the treatment of said disorder. Embodiments of the invention involving measurement of phenotype traits include but need not be

limited to assays of mitochondrial complex V activity, which may further include assays of ATP synthase enzymatic activity and ATP production, assays of reactive oxygen species production, and other assays of electron transport chain activity known to those skilled in the art.

5 After appropriate clinical tests to determine a safe dosage using methods known in the medical and pharmaceutical arts, the test compounds having utility may be administered to humans suffering from or at risk for developing diabetes mellitus. Administration may take place by methods known in the art, *e.g.*, orally, transdermally, by intradermal, intramuscular, subcutaneous, or intravenous injection, *etc.* Treatment
10 with said compounds may prevent or delay the onset of diabetes mellitus, or will serve to treat at least one symptom of the disease.

 Although the present invention is directed primarily towards model systems for diseases in which the mitochondria have metabolic defects, it is not so limited. Conceivably there are disorders wherein there are structural or morphological
15 defects or anomalies, and the model systems of the present invention are of value, for example, to find drugs that address that particular aspect of the disease. In addition, there are certain individuals that have or are suspected of having extraordinarily effective or efficient mitochondrial function, and the model systems of the present invention are of value in studying such mitochondria. In addition, it may be desirable to
20 put known normal mitochondria into cell lines having disease characteristics, in order to rule out the possibility that mitochondrial defects contribute to pathogenesis. All of these and similar uses are within the scope of the present invention, and the use of the phrase "mitochondrial defect" herein should not be construed to exclude such embodiments.

25 **DNA Extraction From Blood Samples**

Blood samples (6-7 ml) from 9 NIDDM patients and 6 non-NIDDM (5 controls and 1 Alzheimer's Disease patient) individuals were collected in EDTA Vacutainer tubes. 6 ml of blood was transferred to a centrifuge tube and 18 ml of dextran solution (3% dextran, average MW = 250,000 kiloDaltons, 0.9% sodium

chloride, 1 mM ethylenedinitrilo tetraacetate) was added and mixed. The tube was maintained at room temperature for 40 minutes without agitation to allow erythrocytes to sediment.

The plasma and leukocyte fraction was transferred to a 15 ml centrifuge tube and leukocytes were collected by centrifugation at 14,000g for 10 minutes. The leukocyte pellet was resuspended in 3.6 ml of water and vortexed for 10 seconds to lyse remaining erythrocytes. 1.2 ml. of 0.6 M sodium chloride was added and the sample was again centrifuged at 14,000g for 10 minutes to collect the leukocytes. The leukocyte pellet was resuspended in 0.4 ml of a solution containing 0.9% sodium chloride/1 mM EDTA, and stored at -80°C.

Total cellular DNA was isolated from 0.2 ml of the frozen leukocyte sample. The frozen leukocytes were thawed, then collected by centrifugation at 12,000g in a microcentrifuge for 5 minutes. The cell pellet was washed once with 0.3 ml of Dulbecco's Phosphate Buffered Saline (PBS: Gibco BRL Life Technologies) and resuspended in 0.2 ml water. The leukocytes were lysed by incubating the sample for 10 minutes at 100°C in a water bath. After the samples were brought to room temperature, cellular debris was pelleted by centrifugation at 14,000g for 2 minutes. The supernatant was transferred to a clean microcentrifuge tube. The DNA concentration was determined by UV absorption at 260 nm.

20 DNA Sequencing

The target tRNA^{Lys} and ATP synthase subunit 8 gene sequences were amplified by polymerase chain reaction (PCR) (Erlich et al., *Nature* 331:461-462 (1988)). Primers were designed using the published Cambridge sequences for normal human mitochondrial genes. (Anderson et al., *Nature* 290:457 (1981)). Three primer pairs were designed with sequences homologous to the tRNA^{Lys} gene and the ATP synthase subunit 6 gene (forward and reverse primers #1 H and #1 L, respectively, Table 1, SEQ ID NO:1 and SEQ ID NO:2), to the cytochrome oxidase subunit 2 gene and the ATP synthase subunit 8 gene (forward and reverse primers #2 H and #2 L, respectively, Table 1, SEQ ID NO:3 and SEQ ID NO:4), and to the tRNA^{Lys} and the

ATP synthase subunit 8 gene (forward and reverse primers #3 H and #3 L, respectively, Table 1, SEQ ID NO:5 and SEQ ID NO:6).

Primers were chemically synthesized using an ABI 394 DNA/RNA synthesizer (Applied Biosystems Division, Perkin Elmer Corp.) using 5 betacyanoethylphosphoramidite chemistry. The primers were deprotected with ammonium hydroxide and purified using Oligonucleotide Purification Cartridges (ABI, Perkin Elmer Corp.).

NIDDM: PCR PRIMERS

SEQ ID NO:	PRIMER	STRAND	LENGTH	POSITION	SEQUENCE 5'→3'
1	Forward Primer	#1L	23mer	8292	GCCCACTGTAAAGCTAACTTAGC
2	Reverse Primer	#1H	22mer	8631	TAGTCGGTTGTTGATGAGATAT
3	Forward Primer	#2L	23mer	8059	CGTCTTGCACTCATGAGCTGTCC
4	Reverse Primer	#2H	25mer	8513	ATTTTCGTTTCATTTTGGTTCTCAGG
5	Forward Primer	#3L	25mer	8311	TAGCATTAAACCTTTTAAGTTAAAGA
6	Reverse Primer	#3H	19mer	8516	TCGTTTCATTTTGGTTCTCA

Table 1

10 Amplification is performed using 0.5-1.0 µg DNA in a reaction volume of 50-100 µl, containing 10 mM Tris HCl (pH 8.3), 50 mM potassium chloride, 2 mM magnesium chloride, 200 µM each of deoxy-ATP, deoxy-CTP, deoxy-GTP and deoxy-TTP (Amplification cocktail), 200 ng each of the appropriate forward and reverse primers and 5 units of AmpliTaq polymerase (Applied Biosystems Division, 15 Perkin Elmer Corp., Foster City, CA; catalogue # N801-0060).

Amplification using primer pairs #1 (SEQ ID NO:1-2) and #2 (SEQ ID NO:3-4) was allowed to proceed for one cycle at 95°C for 10 seconds, 25 cycles at 95°C for 1 minute, 60°C for 1 minute, 72°C for 1 minute, 1 cycle at 72°C for 4 minutes, after which the samples were cooled to 4°C. Amplification using primer pair #3 (SEQ 20 ID NO:5-6) was allowed to proceed for one cycle at 95°C for 10 seconds, 25 cycles at 95°C for 1 min., 50°C for 1 min., 72°C for 1 min., 1 cycle at 72°C for 4 min., after which the samples were cooled to 4°C. Thermocycling reactions are performed using the GeneAmp PCR system 9600 (Applied Biosystems Division, Perkin Elmer Corp.,

Foster City, CA). Four separate amplification reactions are performed for each DNA sample. After the 4 reactions are complete, the reaction products are pooled for each patient and subunit. The pooled product is precipitated at -80°C by the addition of 1/10 volume of 5M sodium chloride and 2 volumes of 100% ethanol.

- 5 The PCR amplification product is pelleted by centrifugation, dried, resuspended in 40 µl of TE buffer and purified by agarose gel electrophoresis. DNA is stained with ethidium bromide and visualized under long-wavelength ultraviolet light. Bands are excised from the gel, minced and placed into a microcentrifuge tube to which 0.3 ml of 1M sodium chloride is added. The tube and contents are frozen at -80°C, 10 thawed and incubated at 40°C for 15-20 minutes. The agarose is sedimented by centrifugation at 14,000 x g for 5 minutes. The supernatant containing the DNA is transferred to a new vial and the DNA is collected by ethanol precipitation.

- The amplified DNA is cloned into plasmid pCRII (Invitrogen Corp., San Diego, CA) using the TA-cloning Kit (Invitrogen Corp., San Diego, CA). 15 Ligations are performed in a reaction volume of 11 µl containing 1-5 µl of PCR amplification product, 2 µl (50 ng) of plasmid, 2 µl of 10x ligation buffer, and 1 µl of T4 DNA ligase (4 units). Ligation reactions are incubated at 10-12°C for 15-16 hours.

- Vector-ligated PCR fragments are transformed into competent *E. coli* cells of the strain XL1-Blue MRF' (Stratagene, San Diego, CA). Transformed cells are 20 spread onto LB-agar plates containing ampicillin (50 mg/ml), kanamycin (50 mg/ml), isopropyl-β-D-thiogalactopyranoside (20 µg/ml), and X-Gal (100 µg/ml). The blue/white color selection provided by the cloning vector allows for easy detection of recombinant clones (*i.e.*, white stained clones).

- Plasmid DNA containing gene inserts is isolated using the Qiawell 96 25 Plasmid Purification Kit (Qiagen, Chatsworth, CA). Plasmid DNA is purified from 5 ml bacterial cultures. The isolated DNA is resuspended in 100 µl TE buffer. The DNA is quantitated by A₂₆₀ absorbance of a 1:50 dilution.

- Sequencing reactions using double stranded plasmid DNA are performed using the Prism™ Ready Reaction DyeDeoxy™ Terminator Cycle Of Sequencing Kit 30 (Applied Biosystems Division, Perkin Elmer Corp., Foster City, CA). The DNA

sequences are detected by fluorescence using the ABI 373A Automated DNA Sequencer (Applied Biosystems Division, Perkin Elmer Corp., Foster City, CA). Oligonucleotide primers are synthesized on the ABI 394 DNA/RNA Synthesizer using standard beta-cyanoethylphosphoramidite chemistry. The following primer sequences were synthesized: M13(-20) forward primer (5'-GTAAAACGACGGCCAG-3', SEQ ID NO:7) and M13 reverse primer (5'-CAGGAAACAGCTATGAC-3', SEQ ID NO:8).

Sequence data were analyzed by comparison with the published Cambridge sequences. (Anderson et al., *Nature* 290:457 (1981)). Mutations for each individual were compiled as summarized in Table 2.

Sequencing reactions were performed according to the manufacturer's instructions. Electrophoresis and sequence analysis are performed using the ABI 373A Data Collection and Analysis Software and the Sequence Navigator Software (Applied Biosystems Division, Perkin Elmer Corp., Foster City, CA). Sequencing gels were prepared according to the manufacturer's specifications. An average of ten different clones from each individual was sequenced. The resulting ATP synthase 8 and tRNA^{Lys} sequences were aligned and compared with the published sequence. Differences in the derived sequence from the published sequence are noted and confirmed by sequence of the complementary DNA strand.

The open reading frame of the mitochondrial gene for ATP Synthase Subunit 8 includes nucleotide positions 8366 to 8572. The open reading frame of the mitochondrial gene for ATP synthase subunit 6 ranges from nucleotide position 8527 through 9204 including overlap of coding sequence at its 5'-end with ATP synthase subunit 8. The gene for the tRNA^{Lys}, which includes nucleotide positions 8295 to 8364, is located directly upstream of the ATP synthase gene.

Clonal analysis of the mitochondrial tRNA^{Lys} gene and ATP synthase 8/6 gene revealed quantitative differences in the levels of heteroplasmy at specific nucleotide positions in these two genes between patients with non-insulin dependent diabetes and controls.

Table 2 shows sequence data using primer pair #1 (SEQ ID NO:1-2) and #2 (SEQ ID NO:3-4) (Table 1) for prior amplification for each of the 15 subjects.

Mutational burden at each specific nucleotide position is indicated as percentage of mutated clones for total quantity of clones sequenced.

Eight base changes were found in the tRNA^{Lys} gene (Table 2). The level of heteroplasmy at each of these nucleotide positions was elevated in most NIDDM patients. Two of the six controls also had modest levels of heteroplasmy at several nucleotide positions.

Twelve nucleotide changes that lead to amino acid changes (missense mutations) were noted in the ATP synthase 8/6 gene. An additional 26 nucleotide changes were seen in the ATP synthase 8/6 gene (Table 2). These additional mutations do not lead to amino acid changes and thus are considered silent mutations. The level of heteroplasmy at each of these nucleotide positions was elevated in most NIDDM patients. Again, two of the six controls also had modest levels of heteroplasmy at some nucleotide positions.

In general, the levels of heteroplasmy at each of these nucleotide sites in these two genes was increased above the controls levels in most patients with NIDDM. For example, the level of heteroplasmy at nucleotide position 8401 varies from 16 to 47% in NIDDM patients and from 0 to 20% in controls. The two controls exhibiting heteroplasmy above 15% may be presymptomatic individuals who are at risk for developing NIDDM. Levels of heteroplasmy above 16% at these specific nucleotide positions may indicate the presence of or risk of developing diabetes mellitus.

Mutational Analysis: tRNA^{Lys} and ATP Synthase Subunits 8/6

			tRNA ^{Lys}										ATP Synthase Subunit 8																			
PATIENT	AG #	DIAGNOSIS																														
			8305	8310	8336	8338	8345	8348	8349	8351	8371	8374	8383	8386	8392	8395	8396	8398	8401	8404	8410	8419	8422	8423	8428	8450						
EO	289	NIDDM	0	0	33	0	33	33	33	33	33	33	33	33	33	33	33	33	33	33	33	33	33	33	33	33						
EP	293	NIDDM	0	0	20	0	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20						
FA	306	NIDDM	0	0	33	0	33	33	33	33	33	33	33	33	33	33	33	33	33	33	33	33	33	33	33	33						
EL	283	NIDDM	8	8	40	7	47	47	47	40	47	47	47	47	47	40	40	40	47	40	47	47	40	40	47	40						
ER	300	NIDDM	9	9	20	0	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20						
469	469	NIDDM	12	12	17	4	21	21	21	17	21	21	21	21	21	17	17	17	21	17	21	17	17	21	17	17						
578	578	NIDDM	15	15	14	2	16	16	16	14	16	16	16	16	16	14	14	14	16	14	16	14	14	16	14	14						
733	733	NIDDM	25	25	4	16	20	20	20	4	20	20	20	20	20	4	4	4	20	4	20	20	4	4	20	4						
909	909	NIDDM	8	8	6	17	23	23	23	6	23	23	23	23	23	6	6	6	23	4	23	23	6	6	23	6						
KJ	294	CONTROL	0	0	10	0	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10						
PS	314	CONTROL	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0						
GM	50	CONTROL	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0						
F8331	331	AD	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0						
GA	27	CONTROL	18	18	2	18	20	20	20	2	20	20	20	20	20	2	2	2	20	2	20	20	2	2	20	20						
SH	25	CONTROL	10	10	8	10	18	18	18	8	18	18	18	18	18	8	8	8	18	8	18	18	8	8	18	18						
			Silent Mutation										Missense Mutation										tRNA Mutation									

Table 2: Part A

Mutational Analysis: tRNA^{Lys} and ATP Synthase Subunits 8/6

ATP Synthase 8/6																					
8459	8463	8467	8470	8473	8474	8485	8488	8487	8488	8491	8503	8506	8508	8509	8512	8539	8541	8557	8562	8566	8568
Asn	Tyr	His	Leu	Pro	Pro	Lys	Pro	Pro	Pro	Met	Asn	Tyr	Asn	Asn	Lys	Ile	Cys	Leu	Pro	Gln	Ser
Asp	Cys	His	Leu	Pro	Thr	Lys	Ser	Leu	Pro	Ile	Asn	Tyr	Ser	Asn	Lys	Ile	Tyr	Leu	Leu	Gln	Tyr
A	A	C	A	T	C	G	C	C	C	A	T	T	A	C	A	C	G	G	C	A	C
C	G	T	G	C	A	A	T	T	T	T	C	C	G	T	G	T	A	A	T	G	A
33	33	33	33	33	33	33	33	33	33	33	33	33	33	33	33	33	40	33	40	33	33
20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20
33	33	33	33	33	33	33	33	33	33	33	33	33	33	33	33	33	33	33	33	33	33
40	40	47	40	47	40	47	40	40	40	47	47	47	40	47	40	40	47	40	47	47	40
20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20
17	17	21	17	21	17	21	17	17	17	21	21	21	17	21	17	17	21	17	21	21	17
14	14	18	14	16	14	18	14	14	14	16	18	18	14	16	14	14	16	14	16	16	14
4	4	20	4	20	4	20	4	4	4	20	20	20	4	20	4	4	20	4	20	20	4
6	6	23	6	23	6	23	6	6	6	23	23	23	6	23	6	6	23	6	23	23	6
10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2	2	20	2	20	2	20	2	2	2	20	20	20	2	20	2	2	20	2	20	20	2
8	8	18	8	18	8	18	8	8	8	18	18	18	8	18	8	8	18	8	18	18	8

Table 2: Part B

Synthesis of Antisense Oligonucleotides

Standard manufacturer protocols for solid phase phosphoramidite-based DNA or RNA synthesis using an ABI DNA synthesizer are employed to prepare antisense oligomers. Phosphoroamidite reagent monomers (T, C, A, G, and U) are used as received from the supplier. Applied Biosystems Division/Perkin Elmer, Foster City, CA. For routine oligomer synthesis, 1 μ mole scale synthesis reactions are carried out utilizing THF/ I_2 /lutidine for oxidation of the phosphoramidite and Beaucage reagent for preparation of the phosphorothioate oligomers. Cleavage from the solid support and deprotection are carried out using ammonium hydroxide under standard conditions. Purification is carried out via reverse phase HPLC and quantification and identification is performed by UV absorption measurements at 260 nm, and mass spectrometry.

Inhibition of Mutant Mitochondria in Cell Culture

Antisense phosphorothiodate oligomer complementary to the ATP synthase and tRNA^{Lys} gene mutants and thus non-complementary to wild-type ATP synthase or tRNA^{Lys} genes are added to fresh medium containing Lipofectin® Gibco BRL (Gaithersburg, MD) at a concentration of 10 μ g/ml to make final concentrations of 0.1, 0.33, 1, 3.3, and 10 μ M. These are incubated for 15 minutes then applied to the cell culture. The culture is allowed to incubate for 24 hours and the cells are harvested and the DNA isolated and sequenced as in previous examples. Results of quantitative analysis show a decrease in mutant ATP synthase or tRNA^{Lys} DNA to a level of less than 1% of total ATP synthase or tRNA^{Lys} wild-type DNA.

Vehicle and nonsense oligonucleotide controls show no decrease in mutant ATP synthase or tRNA^{Lys} DNA.

Inhibition of Mutant Mitochondria *In Vivo*

Mice are divided into six groups of 10 animals per group. The animals are housed and fed as per standard protocols. Antisense phosphorothioate oligonucleotide complementary to mutant ATP synthase gene RNA, prepared as

described above, is administered intramuscularly (I.M.) to groups 1 to 4, in the following amount: 0.1, 0.33, 1.0 and 3.3 nmol each in 5 μ L. To group 5 is administered I.M. 1.0 nmol in 5 μ L of phosphorothioate oligonucleotide non-complementary to mutant ATP synthase gene RNA and non-complementary to wild-type ATP synthase gene RNA. To group 6 is administered I.M. vehicle only. Dosing is performed once a day for ten days. The animals are sacrificed and samples of muscle and pancreas collected. This tissue is treated as previously described and the DNA isolated and quantitatively analyzed as in previous examples. Results show a decrease in mutant ATP synthase DNA to a level of less than 1% of total ATP synthase for the antisense treated group and no decrease for the control group.

Agents for the Detection and Selective Destruction of Defective Mitochondria

a. Preparation of 10-N-(10-Hydroxy-1-decyl)-3,6 bis(dimethylamino)acridine bromide salt

3,6-bis(dimethylamino)acridine (1.0 millimole) is dissolved in DMF (100 ml) containing 1.1 equivalent of tertiary amine base. To this is added 10-hydroxy-1-bromo decane (1.1 millimole), and the mixture is heated to reflux. When monitoring by TLC shows no remaining 3,6bis(dimethylamino)acridine, the reaction is cooled and the 10-N-(10-hydroxy-1-decyl)-3,6-bis(dimethylamino)acridine is isolated (0.75 millimoles).

b. Preparation of 10-N-(10-phosphoryl-1-decyl)-3, 6-bis(dimethylamino)acridine chloride salt

10-N-(10-Hydroxy-1-decyl)-3,6-bis(dimethylamino)acridine (1.0 millimole) is dissolved in pyridine (100 ml). To this is added 2-(N,N-dimethylamino)-4-nitrophenyl phosphate (1.1 millimole) according to the procedure of Taguchi (*Chem. Pharm. Bull.* 23:1586 (1975)), and the mixture is stirred under a nitrogen atmosphere. When monitoring by TLC showed no remaining 10-N-(10-hydroxy-1-decyl)-3,6-bis(dimethylamino)acridine, the reaction is worked up according to Taguchi and the

10-N-(10-phosphoryl-1-decyl)-3,6-bis(dimethylamino)acridine is isolated (0.75 millimoles).

c. Preparation of 10-N-(10-thiophosphoryl-1-decyl)-3,6-bis(dimethylamino)acridine chloride salt

- 5 10-N-(10-hydroxy-1-decyl)-3,6-bis(dimethylamino)acridine (1.0 millimole) is dissolved in DMF (100 ml). To this is added triimidazolyl-1-phosphine sulfide (1.1 millimole) according to the procedure of Eckstein (*Journal of the American Chemical Society* 92:4718, (1970)) and the mixture stirred under a nitrogen atmosphere. When monitoring by TLC shows no remaining 10-N-(10-Hydroxy-1-decyl)-3,6-bis(dimethylamino)acridine, the reaction is worked up according to Eckstein and the
- 10 10-N-(10-thiophosphoryl-1-decyl)-3,6-bis(dimethylamino)acridine is isolated (0.75 millimoles).

d. Preparation of 10-N-(11-undecanoic acid)-3,6-bis(dimethylamino)acridine bromide salt

- 15 3,6-Bis(dimethylamino)acridine (1.0 millimole) is dissolved in DMF (100 ml). To this is added 11-bromo undecanoic acid (1.1 millimole) and the mixture is heated to reflux. When monitoring by TLC shows no remaining 3,6-bis(dimethylamino)acridine, the reaction is cooled and the 10-N-(11-undecanoic acid)-3,6-bis(dimethylamino)acridine is isolated (0.75 millimoles).

20 e. Preparation of 10-N-(11-undecyl-2,4-dinitrophenyl urethane)-3,6-bis(dimethylamino)acridine bromide salt

- 10-N-(11-Undecanoic acid)-3,6-bis(dimethylamino)acridine (1.0 millimole) is dissolved in DMF (100 ml). To this is added 2,4-dinitrophenol (1.1 millimole) and diphenylphosphoryl azide (1.1 millimole), and the mixture is stirred
- 25 while heating to 70°C. When monitoring by TLC shows no remaining 10-N-(11-undecanoic acid)-3,6-bis(dimethylamino)-acridine, the reaction is cooled and

the 10-N-(11-undecyl-2,4-dinitrophenyl urethane)-3,6bis(dimethylamino)acridine is isolated (0.75 millimoles).

f. Preparation of 10-N-(11-undecan-1-oic acid 2,4-dinitrophenyl ester)-3,6-bis(dimethylamino)acridine bromide salt

5 10-N-(11-undecanoic acid)-3,6-bis(dimethylamino)acridine (1.0 millimole) is dissolved in DMF (100 ml). To this is added 2,4-dinitrophenol (1.1 millimole), dicyclohexylcarbodiimide (1.1 millimole) and hydroxybenzotriazole (1.1 millimole), and the mixture is stirred. When monitoring by TLC shows no remaining 10-N-(11-undecanoic acid)-3,6-bis(dimethylamino)-acridine, the reaction is cooled and
10 the 10-N-(11-undecan-1-oic acid 2,4-dinitrophenyl ester)-3,6-bis(dimethylamino)acridine is isolated (0.75 millimoles).

g. Preparation of N'-(2-hydroxyethyl)-JC-1

According to the procedure of Yamamoto et al., *Bulletin of the Chemical Society of Japan* 46:1509-11 (1973)), 2-methyl-5,6-dichloro-N-ethyl-N'-(2-hydroxyethyl) benzimidazole is heated with aniline and ethyl orthoformate
15 at 100°C. To this is added acetic anhydride and potassium acetate and heating is continued at 160°C. The reaction is worked up as described in Yamamoto et al. and the product isolated.

h. Preparation of bis N'-(2-phosphoryl-1-ethyl)-JC-1

20 N'-(2-hydroxyethyl)-JC-1 (1.0 millimole) is dissolved in pyridine (100 ml). To this is added 2-(N,N-dimethylamino)-4-nitrophenyl phosphate (1.1 millimole) according to the procedure of Taguchi, *Chem. Pharm. Bull.* 23:1586 (1975), and the mixture is stirred under a nitrogen atmosphere. When monitoring by TLC shows no remaining 10-N-(10-hydroxy-1-decyl)-3,6bis(dimethylamino)acridine, the reaction is
25 worked up according to Taguchi and bis N'-(2-phosphoryl-1-ethyl) JC-1 was isolated (0.75 millimoles).

Generation of Cybrid Cells

The human neuroblastoma cell line SH-SY5Y was depleted of mitochondrial DNA by prolonged culture in the presence of ethidium bromide to yield ρ^0 cells, as previously described. (Miller et al., *J. Neurochem.* 67:1897 (1996)) To produce mitochondrial cybrid cell lines from the ρ^0 cells, blood-derived platelets were used as sources of donor mitochondria. Platelet-enriched buffy coat fractions were isolated from fresh venous blood donated by normal and diabetic patients, and were then fused with the ρ^0 -SH-SY5Y cells. In this way, mitochondrial DNA-encoded subunits of the electron transport complexes and ATP synthase in each cybrid cell line are transcribed entirely from the mitochondrial DNA donated by a patient's mitochondria. Using this technique, cell lines that express mitochondrial populations from individual normal donors or diabetic patients were constructed. Several analyses were performed to assess the phenotype (function) and genotype (DNA sequences) of the mitochondria in these cell lines.

15 Reactive Oxygen Species Production in Cybrid Cells Derived from Diabetic Patient Mitochondria

Production of reactive oxygen species (ROS) was measured in cybrid cell lines as a general indicator of mitochondrial function. Cybrid cell lines produced using mitochondria from normal (wild-type control) or NIDDM patients were grown in 96-well plates and then incubated in the presence of the fluorescent dye 2',7'-dichlorodihydrofluorescein diacetate (DCFC, 30 μ M; Molecular Probes, Eugene, OR) in Hanks balanced salt solution (HBSS) for 2 hr at 37°C. (Miller et al., *J. Neurochem.* 67:1897 (1996)) The cells were washed twice with HBSS, and the fluorescence was read 30 min later at an excitation wavelength of 485 nm, emission wavelength 530 nm in a Cytofluor fluorescence plate reader. ROS production was proportional to the increase in DCFC fluorescence in the cells.

A striking increase in the generation of ROS was observed in the NIDDM cybrids when compared to normal control cybrid cells (Figure 6). Since the control and NIDDM cybrids differ only in their mitochondrial genomes, this finding

suggested that NIDDM cybrids express a genetic defect in one or more mitochondrial encoded peptides.

Mitochondrial Enzyme Activity in Cybrid Cells Derived from Diabetic Patient Mitochondria

5 Catalytic activities of three distinct mitochondrial enzyme complexes were measured in cybrid cell lines produced using NIDDM or normal (wild-type control) patient-derived mitochondria. At least some of the constituent enzyme subunits in each of these mitochondrial protein complexes are encoded by mitochondrial genes. Defective catalytic activity in any of these complexes suggests
10 the specific site of diabetes-associated defective mitochondrial function.

Cybrid cell lines grown in culture were harvested by trypsinization and lysed by the addition of digitonin (0.005% w/v) and EDTA (5 mM) in HBSS for 20 sec at 23°C. Fifty volumes HBSS were then added and the suspension centrifuged at 14,000 x g for 10 min at 4°C. The pellet was resuspended in HBSS containing EDTA
15 (5 mM), leupeptin (1 µM), pepstatin (1 µM) and phenylmethylsulfonyl fluoride (100 µM) at a final protein concentration of 3-6 mg/ml. A 200 µl aliquot of the resultant submitochondrial particulate fraction ("sample") was sonicated for 6 min at 50% duty cycle (w/v) at 50% power in an ice-packed cup horn sonicator (Branson Sonifier 450, Danbury, CT) immediately prior to assay.

20 Complex I (NADH:ubiquinone oxidoreductase) activity was measured by sequentially adding NADH (100 µM final concentration), 100 µg/ml sample protein, and coenzyme Q₁ (Eisai Pharmaceuticals, Tokyo, Japan; 42 µM final concentration) to prewarmed 30°C assay buffer (25 mM potassium phosphate, pH 8.0, 0.25 mM EDTA, 1.5 mM potassium cyanide). The absorbance change at 340 nm was monitored
25 spectrophotometrically for 2 min, after which 2.5 µM rotenone was added for an additional 2 min.

Complex IV (cytochrome c oxidase) activity was measured as described (Parker et al., *Neurology* 40:1302-1303 (1990)) except that sample submitochondrial particulate fractions were prepared as described above and assay reaction volumes were

reduced. Assay reactions were initiated by addition of reduced cytochrome c to spectrophotometer cuvettes containing sample aliquots, and the change in absorbance at 550 nm was measured continuously for 90 sec. The fully oxidized absorbance value was determined by the addition of a few grains of ferricyanide to the cuvette. Rates
5 were obtained at various sample concentrations to validate that the assay was in the linear range. Non-enzymatic background activity was determined by preincubation of sample with 1 mM potassium cyanide prior to determination of the rate constant.

Complex V (ATP synthase) activity was determined by incubating samples containing 1 mg protein with 1 mM ATP, 0.3 mM NADH, 10 U/ml LDH, 1
10 mM phosphoenolpyruvate, and 2.5 U/ml pyruvate kinase in 50 mM Tris-HCl buffer, pH 8.0 at 30°C in a total volume of 1 ml. The change in absorbance at 340 nm was monitored spectrophotometrically for 10 min, and all activities were normalized to total cellular protein.

The activities of complex I and complex IV were not different in
15 submitochondrial particles prepared from normal control and NIDDM cybrid cell lines (Table 3), indicating that mutations of mitochondrial genes encoding complex I and complex IV subunits are not likely responsible for the mitochondrial dysfunction manifested as elevated ROS production in NIDDM cybrids.

In contrast, complex V (ATP synthase) activity was decreased 35% in
20 NIDDM cybrids when compared to control cybrids.

These results suggest that decreased ATP synthase activity is responsible for the observed mitochondrial dysfunction, namely increased ROS production. To test the alternative hypothesis, *i.e.*, that increased ROS generation could somehow cause decreased ATP synthase activity, complex V activity was measured in cybrids
25 generated using mitochondria from Alzheimer's Disease (AD) patients. Like the NIDDM cybrids, AD cybrids produced increased ROS compared to normal (wild-type) control cybrids. However, ATP synthase activity levels in AD cybrids were comparable to those in normal control cybrids. (data not shown) Thus decreased cybrid cell ATP synthase activity is not a consequence of excessive ROS production, but instead appears
30 to be causally related to ROS generation (Figure 6).

Table 3

Cybrid	Complex I ($\mu\text{M}/\text{min}/\text{mg}$)	Complex IV ($\text{min}^{-1} \text{mg}^{-1}$)	Complex V (ATP Synthase) ($\text{nmol}/\text{min}/\text{mg}$)
Control	27.5 ± 1.2	2.05 ± 0.06	17.1 ± 1.2
NIDDM	26.6 ± 0.84	1.92 ± 0.29	11.2 ± 0.88

NIDDM Patients Exhibit Increased Mutational Burden of Mitochondrially

5 Encoded ATP Synthase 8/6 Genes

Mitochondrial DNA from NIDDM and non-diabetic (control) patients was compared to quantify the percentage of mutant DNA molecules (% heteroplasmy). Venous blood samples were collected from 24 NIDDM patients (age = 49.8 ± 4.6 ; body mass index, BMI = $33.3 \text{ kg}/\text{m}^2 \pm 0.83$; 67% male, 33% female) and 15 age and weight-

10 matched controls (age = 47.3 ± 2.1 ; BMI = 31.6 ± 1.0 ; 73% male, 27% female). The ATP synthase 8 gene was isolated and sequenced as described above. Fifty clones were analyzed for each patient sample.

The percentage of mutant mitochondrial DNA molecules (Figure 7) was almost twice as great ($p < 0.001$) in the NIDDM subjects compared to the age- and

15 weight-matched non-diabetic controls.

NIDDM Cybrid Cells Contain Mutated Mitochondrial Genomes

Defective mitochondrial function, and specifically defective ATP synthase 8 activity, were observed in cybrid cells generated by fusing ρ^0 cells with platelets derived from NIDDM patients, as described above. To demonstrate the

20 presence of the mutant mitochondrial genome in these cybrid cells, and more particularly to show that a mutated ATP synthase 8 gene was present in these cells, DNA from a NIDDM cybrid cell line was prepared and analyzed by slot blot hybridization.

Total cellular DNA was extracted from a NIDDM cybrid cell line and from ρ^0 cells. One microgram of DNA was loaded into each slot of a slot blot apparatus. Duplicate slot blots were probed with [^{32}P]-labeled oligonucleotide probes specific for wild-type or mutant ATP synthase 8 gene sequences under hybridization and washing conditions that produced $\geq 97\%$ specificity for each probe. The wild-type probe was a fragment corresponding to base pairs 8831-8536 in the wild-type genome (Anderson et al., *Nature* 290:457 (1981)), while the mutant probe was a fragment corresponding to base pairs 8326-8526 in the mutant mitochondrial genome, and contained the point mutations presented in Table 2.

10 No mitochondrial DNA signal was detected in ρ^0 cells. The mutant ATP synthase 8/6 genome was readily detectable in the NIDDM cybrid-derived DNA. Quantitation of the blots revealed that these diabetic cells were approximately 20% heteroplasmic. This observation confirms that the mutant ATP synthase 8/6-containing genome was successfully transferred into the NIDDM cybrid cells along with the donor patients' mitochondria. This result also demonstrates heteroplasmy in a NIDDM patient mitochondrial sample, since both wild-type and mutant ATP synthase 8/6 sequences were detected.

20 From the foregoing it will be appreciated that the description of the invention and the various embodiments thereof is intended to be illustrative and not limiting. Although the invention has been described with reference to the above-provided examples, it should be understood that various modifications can be made without departing from the spirit of the invention.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: Mitokor
- (ii) TITLE OF INVENTION: MITOCHONDRIAL DNA MUTATIONS THAT SEGREGATE WITH LATE ONSET DIABETES MELLITUS
- (iii) NUMBER OF SEQUENCES: 8
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: SEED and BERRY LLP
 - (B) STREET: 6300 Columbia Center, 701 Fifth Avenue
 - (C) CITY: Seattle
 - (D) STATE: Washington
 - (E) COUNTRY: USA
 - (F) ZIP: 98104
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.30
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER: US
 - (B) FILING DATE: 21-OCT-1997
 - (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: Maki, David J.
 - (B) REGISTRATION NUMBER: 31,392
 - (C) REFERENCE/DOCKET NUMBER: 660088.406PC
- (ix) TELECOMMUNICATION INFORMATION:
 - (A) TELEPHONE: (206) 622-4900
 - (B) TELEFAX: (206) 682-6031

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 23 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: double
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GCCCACTGTA AAGCTAAGTT AGC

23

(2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 22 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TAGTCGGTTG TTGATGAGAT AT

22

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 23 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CGTCTTGCAC TCATGAGCTG TCC

23

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 25 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

ATTTTCGTTC ATTTTGTTTC TCAGG

25

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 25 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

TAGCATTAAAC CTTTTAAGTT AAAGA

25

(2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 19 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: double
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

TCGTTTCATTT TGGTTCTCA

19

(2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 16 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GTAAAACGAC GGCCAG

16

(2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 17 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CAGGAAACAG CTATGAC

17

CLAIMS

What is claimed is:

1. A method for detecting the presence or risk of developing diabetes mellitus in a human, said method comprising:

determining the presence in a biological sample from a human of at least one mitochondrial mutation in an ATP synthase gene which correlates with the presence of or risk of developing diabetes mellitus.

2. A method according to claim 1 wherein said mutation is in an ATP synthase gene at nucleotide position 8371, 8374, 8383, 8386, 8392, 8395, 8396, 8398, 8401, 8404, 8410, 8419, 8422, 8423, 8428, 8450, 8459, 8463, 8467, 8470, 8473, 8474, 8485, 8486, 8487, 8488, 8491, 8503, 8506, 8508, 8509, 8512, 8539, 8541, 8557, 8562, 8566, 8568 or combinations thereof.

3. The method of claim 2 wherein said at least one mutation is a silent mutation, missense mutation, or combination thereof.

4. A method for detecting the presence or risk of developing diabetes mellitus in a human, said method comprising:

determining the presence in a biological sample from a human of at least one mitochondrial mutation in a tRNA^{Lys} gene which correlates with the presence of or risk of developing diabetes mellitus.

5. The method of claim 4 wherein said at least one mutation which correlates with the presence or risk of developing diabetes mellitus is a tRNA^{Lys} gene at nucleotide position 8336, 8345, 8348, 8349, 8351 or a combination thereof.

6. The method according to claims 1 or 4 wherein the presence of at least one mitochondrial mutation which correlates with the presence or risk of developing diabetes mellitus is determined by a technique that is selected from the group of techniques consisting

of: hybridization with oligonucleotide probes, a ligation reaction, a polymerase chain reaction and variations thereof and single nucleotide primer-guided extension assays.

7. A method of detecting genetic mutations which cause diabetes mellitus or indicate a predisposition to develop diabetes mellitus, said method comprising:

- a) determining the sequence of at least one mitochondrial ATP synthase gene from humans known to have diabetes mellitus;
- b) comparing said sequence to that of the corresponding wildtype mitochondrial ATP synthase gene; and
- c) identifying mutations in said humans which correlate with the presence of diabetes mellitus.

8. An isolated nucleotide sequence which is at least partially complementary to a mitochondrial DNA sequence, wherein said isolated sequence contains at least one mutation in an ATP synthase subunit 8/6 gene which correlates with the presence or risk of diabetes mellitus.

9. An isolated nucleotide sequence which is at least partially complementary to a mitochondrial DNA sequence, wherein said isolated sequence contains at least one mutation in a tRNA^{Lys} gene which correlates with the presence or risk of diabetes mellitus.

10. An isolated nucleotide sequence which is at least partially complementary to a mitochondrial DNA sequence, wherein said isolated sequence contains at least one mutation located between mitochondrial DNA nucleotides 8295 and 8571 which correlates with the presence or risk of diabetes mellitus.

11. The isolated nucleotide sequence of any one of claims 8-10 wherein said isolated sequence is labelled with a detectable agent.

12. A method of inhibiting the transcription or translation of one or more mutant ATP synthase encoding nucleic acids correlated with late onset diabetes mellitus, or the transcription of one or more mutant tRNA^{Lys} encoding nucleic acids correlated with late onset diabetes mellitus, said method comprising:

- a) contacting said ATP synthase or tRNA^{Lys} encoding nucleic acids with antisense sequences specific to said mutant nucleic acids; and
- b) allowing hybridization between target mutant ATP synthase or tRNA^{Lys} encoding nucleic acids and said antisense sequences.

13. A method according to claim 12 wherein hybridization is performed under conditions wherein the antisense sequences bind to and inhibit transcription or translation of target mutant ATP synthase 8/6 encoding nucleic acid or inhibit transcription of target mutant tRNA^{Lys} encoding nucleic acid without preventing transcription or translation of the corresponding wild-type nucleic acids or other mitochondrial genes.

14. The method of claim 12 wherein said mutant ATP synthase or tRNA^{Lys} encoding nucleic acids are RNA.

15. A method for evaluating a compound for use in diagnosis or treatment of diabetes mellitus, said method comprising:

- a) contacting a predetermined quantity of said compound with cultured cybrid cells having genomic DNA originating from a ρ^0 cell line and mutant mitochondrial DNA originating from tissue of a human having a disorder that is associated with late onset diabetes mellitus;
- b) measuring a mitochondrial complex V activity that is affected by said mutant mitochondrial DNA in said cybrid cells; and
- c) correlating a change in the mitochondrial complex V activity with effectiveness of the compound.

16. A method according to claim 15 wherein the ρ^0 cell line is immortal.

17. A method for evaluating a compound for its utility in the diagnosis and treatment of diabetes mellitus, said method comprising:

- a) inducing differentiation of cultured undifferentiated cybrid cells having genomic DNA originating from a ρ^0 cell line and mutant mitochondrial DNA originating from tissue of a human having a disorder that is associated with late onset diabetes mellitus;
- b) contacting a predetermined quantity of said compound with the cybrid cells of step (a);
- c) measuring a mitochondrial complex V activity that is affected by mutant mitochondrial DNA in said cybrid cells of step b); and
- d) correlating a change in the mitochondrial complex V activity with effectiveness of the compound in the diagnosis or treatment of diabetes mellitus.

18. A method according to claim 17 wherein said ρ^0 cell line is immortal.

19. A method for detecting the presence of diabetes mellitus in a human subject, comprising the steps of:

- a) obtaining a biological sample containing mitochondria from said subject; and
- b) determining the presence of at least one polypeptide encoded by a mitochondrial ATP synthase gene.

20. The method of claim 19 wherein the presence of said polypeptide is determined with at least one monoclonal antibody or polyclonal antibody.

21. A method for treating an individual afflicted with diabetes mellitus comprising selectively introducing into the mitochondria of said individual a conjugate molecule, comprising a targeting molecule conjugated to a toxin, therapeutically useful agent, or an imaging ligand via a linker, wherein the targeting molecule recognizes a mutant mitochondrial ATP synthase 8/6 or tRNA^{Lys} gene or its transcript or expressed product.

22. The method of claim 27 wherein said targeting molecule is selected from the group consisting of antibodies, nucleic acids, proteins and small molecules.

23. The method of claim 21 wherein said linker contains a functional group selected from ester, ether, thioether, phosphorodiester, thiophosphorodiester, carbonate, carbamate, hydrazone, oxime, amino and amide.

24. The method of claim 21 wherein said imaging ligand is selected from the group consisting of radioisotopes, haptens, biotin, enzymes, fluorophores and chemilumiphores.

25. The method of claim 21 wherein said toxin is selected from the group consisting of phosphate, thiophosphate, dinitrophenol, maleimide and antisense oligonucleic acids.

26. The method of claim 21 wherein said therapeutically useful compounds are antioxidants or free radical trapping agents.

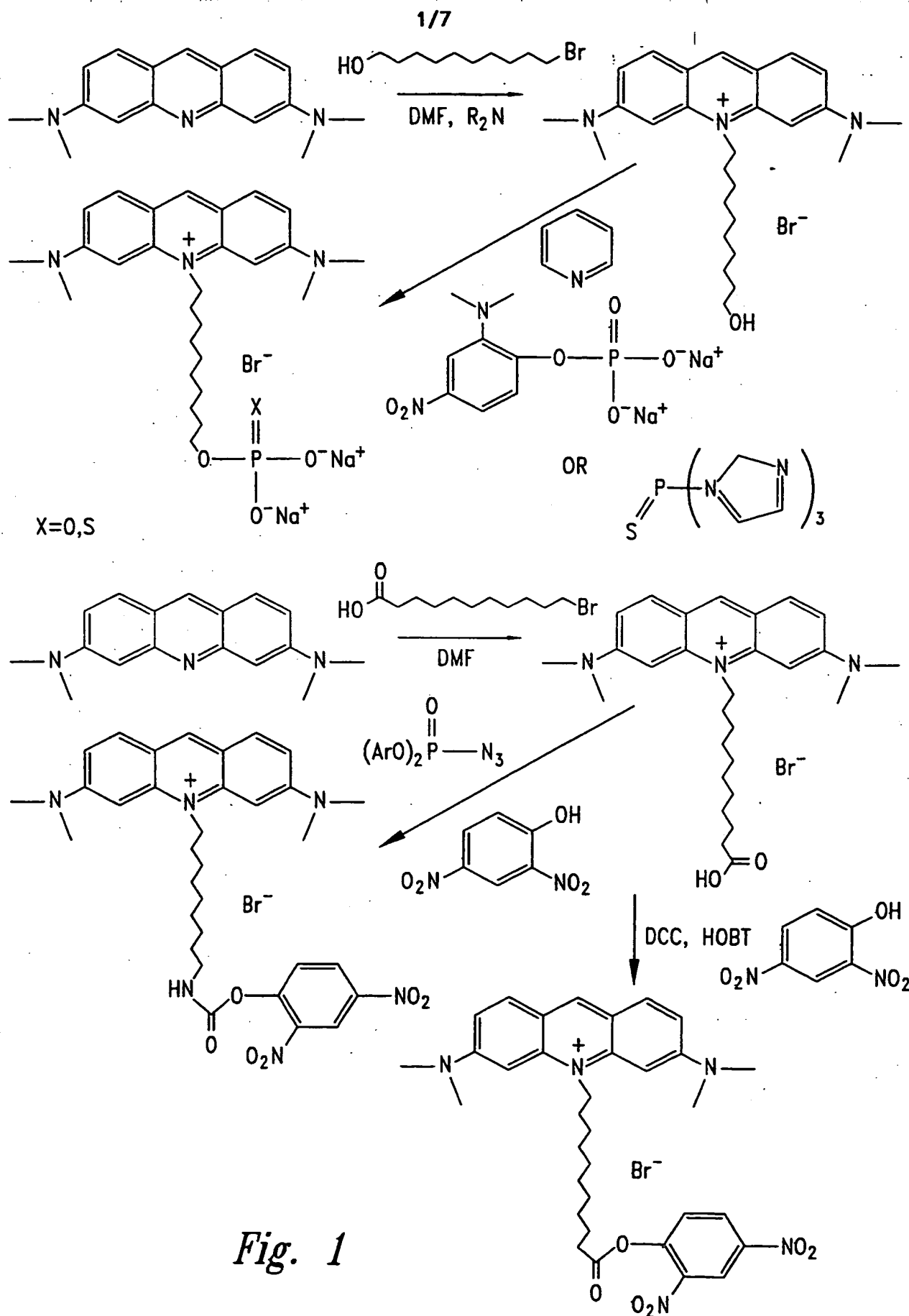
27. A method for detecting the presence or risk of developing diabetes mellitus in a human, said method comprising:

determining the presence in a biological sample from a human of at least one mitochondrial mutation which correlates with the presence of or risk of developing diabetes mellitus.

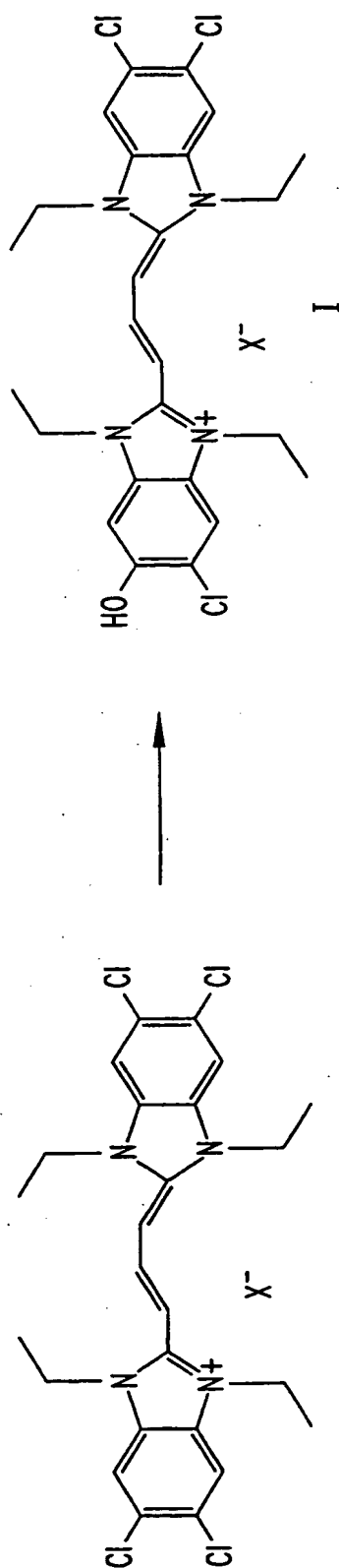
28. An isolated nucleotide sequence which is at least partially complementary to a mitochondrial DNA sequence, wherein said isolated sequence contains at least one mutation that correlates with the presence or risk of diabetes mellitus.

29. A method for evaluating a compound for use in diagnosis or treatment of diabetes mellitus, said method comprising:

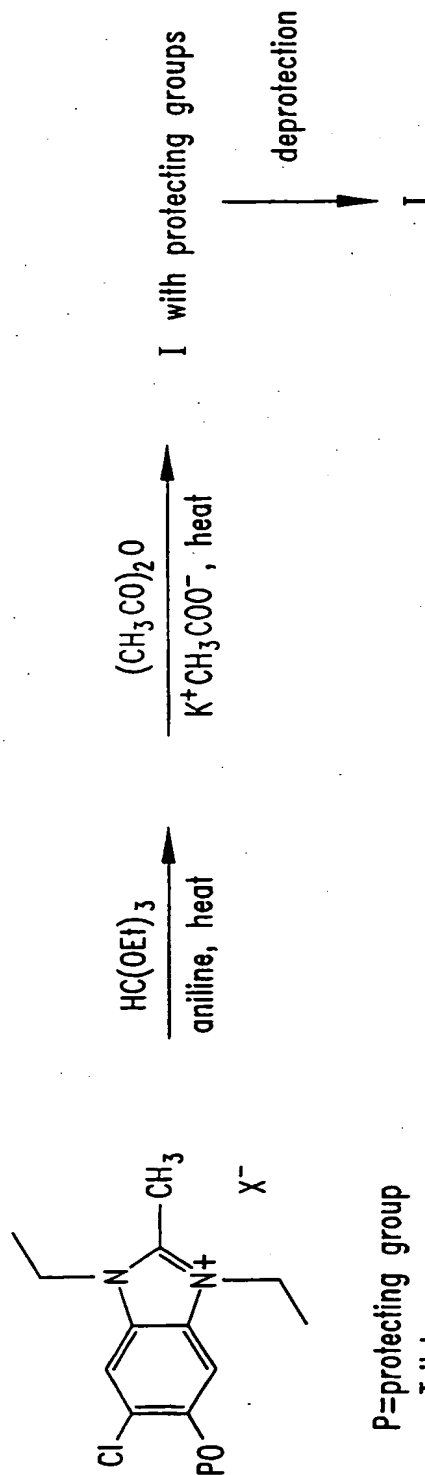
- a) contacting a predetermined quantity of said compound with cultured cybrid cells having genomic DNA originating from a ρ^0 cell line and mutant mitochondrial DNA originating from tissue of a human having a disorder that is associated with late onset diabetes mellitus;
- b) measuring a phenotypic trait in said cybrid cells that is affected by said mutant mitochondrial DNA; and
- c) correlating a change in the phenotypic trait with effectiveness of the compound.



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Converting chloro to heteroatom to provide attachment points for drug molecules
 Heteroatom could be O, N, S



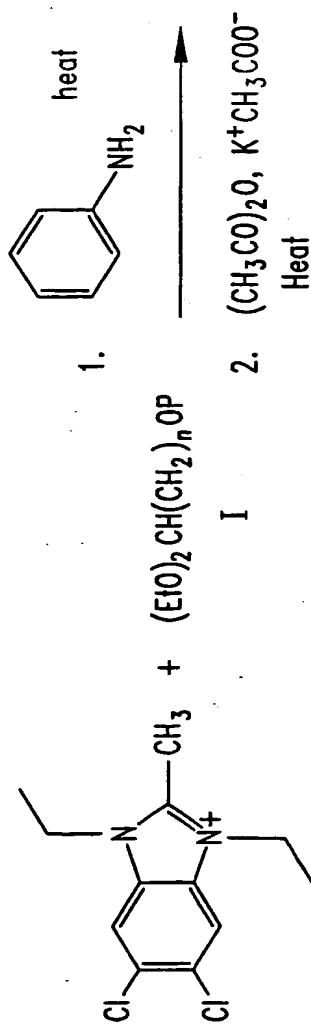
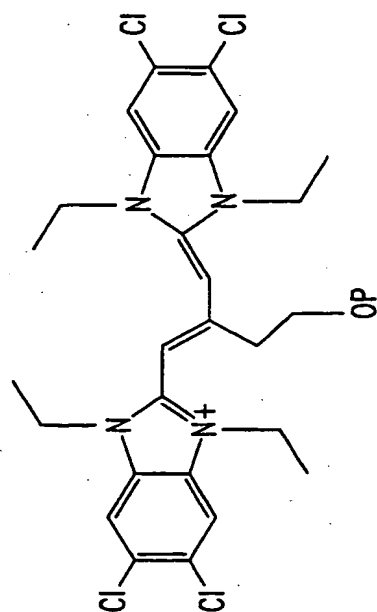
P=protecting group

=Trityl

=Si(R)₃ where R = alkyl

Fig. 2

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The spacer in I could be varied in length such that $n = 1$ to 10

Fig. 3

4/7

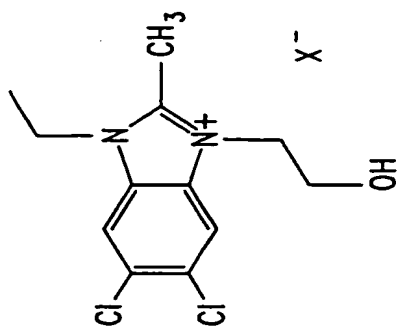
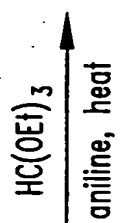
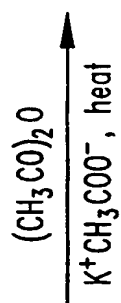
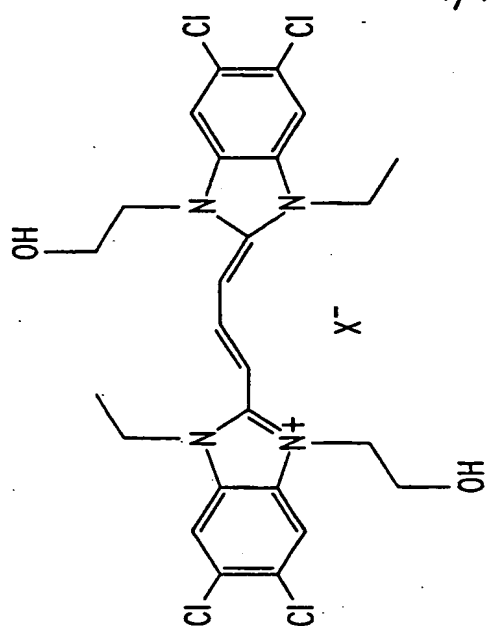
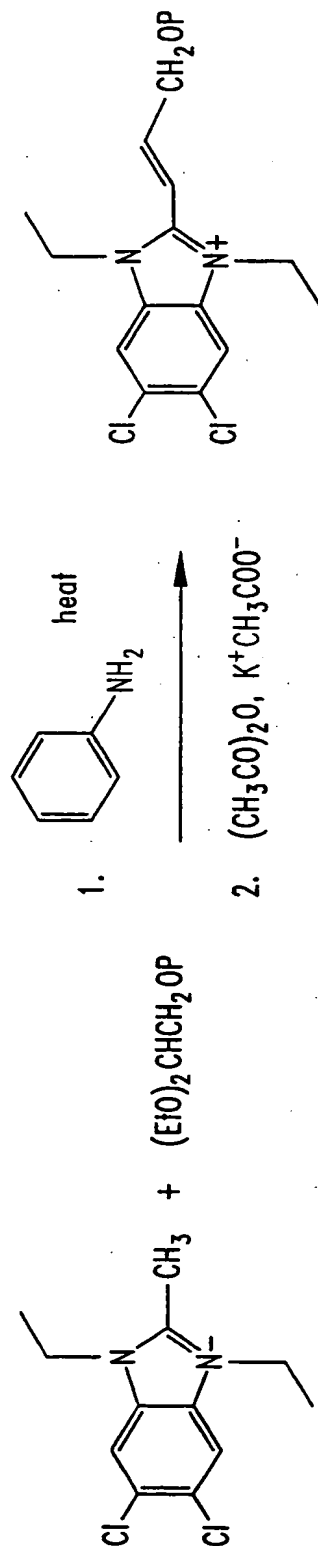


Fig. 4

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P=protecting group

=Si(R)₃ where R = alkyl

=Trityl

other possible linking units:

 $(EtO)_2CH-CH_2COOP$ $(EtO)_2CH-CH_2NHP$ $(EtO)_2CH-CH_2SP$ $(EtO)_2CH-(CH_2)_n-X$

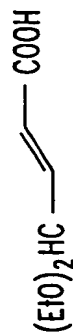
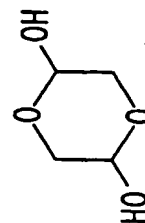
n=2-10

x=OH

=NH₂

=SH

=COOH



n=2-10

x=OH

=NH₂

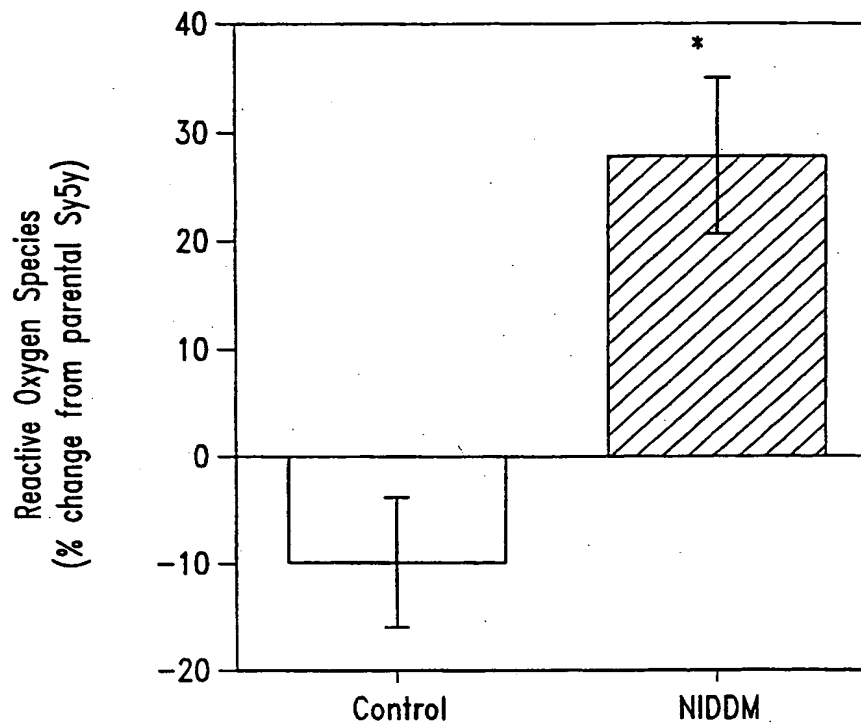
=SH

=COOH

Fig. 5

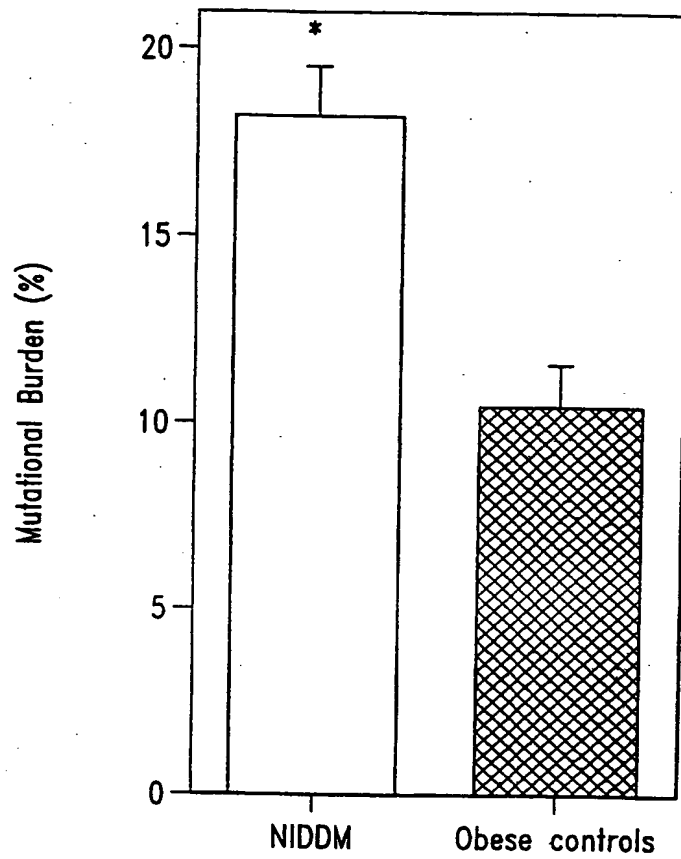
For each acetal represented, this could also be a ketal where H is replaced by an alkyl group C1 to C10

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*Fig. 6*

SUBSTITUTE SHEET (RULE 26)

7/7

*Fig. 7*

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 97/19023

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EGAWHARY D N ET AL.: "Diabetic complications and the mechanism of the hyperglycemia-induced damage to the mt DNA of cultured vascular endothelial cells: (I). Characterization of the 4977 base pair deletion and 13 bp flanking repeats." BIOCHEMICAL SOCIETY TRANSACTIONS, vol. 23, no. 4, 1995, page 518S XP002052327 see the whole document ---	1-3,6-8, 10,27,28
X	WALLACE D C : "Mitochondrial genetics: A paradigm for aging and degenerative diseases ?" SCIENCE, vol. 256, 1992, pages 628-632, XP002052328 see the whole document ---	1,4,5, 7-10,27, 28
- / - -		

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- *Z* document member of the same patent family

Date of the actual completion of the international search

16 January 1998

Date of mailing of the international search report

13.02.98

Name and mailing address of the ISA
European Patent Office, P.B. 5818 Patentlaan 2
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Fax: (+31-70) 340-3016

Authorized officer

Knehr, M

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 97/19023

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SUZUKI S ET AL.: "Diabetes with mitochondrial gene tRNALYS mutation." DIABETES CARE, vol. 17, no. 12, 1994, pages 1428-1432, XP002052329 see the whole document ---	4-6, 9-11,27, 28
X	WO 91 19815 A (WALLACE DOUGLAS C) 26 December 1991 see abstract see page 7, line 1 - line 15 see page 9, line 36 - page 10, line 12 see page 11, line 2 see page 19, line 13 - line 14 see page 22, line 1 - line 29; claims 1-29; figures 1-3 ---	8-11,28
X	MATHEWS C E ET AL.: "A point mutation in the mitochondrial DNA of diabetes-prone BHE/cdb rats." FASEB JOURNAL, vol. 9, 1995, pages 1638-1642, XP002052330 see the whole document ---	7,8,28
X	LUFT R: "The development of mitochondrial medicine" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES USA, vol. 91, 1994, pages 8731-8738, XP002052331 see the whole document ---	27,28
X	WO 95 26973 A (APPLIED GENETICS INC ;HERRNSTADT CORINNA (US); PARKER WILLIAM DAVI) 12 October 1995 see the whole document ---	27-29
Y	see the whole document ---	1-22,26
X	JOHNS D R: "Mitochondrial DNA and disease" THE NEW ENGLAND JOURNAL OF MEDICINE, vol. 333, no. 10, 1995, pages 638-644, XP002052332 see the whole document ---	27,28
Y	see the whole document ---	1-22,26
X	GERBITZ K D ET AL.: "Mitochondria and diabetes. Genetic, biochemical, and clinical implications of the cellular energy circuit." DIABETES, vol. 45, no. 2, 1996, pages 113-126, XP002052333 see the whole document ---	4-6,9, 10,27,28
Y	see the whole document ---	15-20
	--/--	

INTERNATIONAL SEARCH REPORT

Interr. Int'l Application No.

PCT/US 97/19023

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	<p>SWERDLOW R H ET AL.: "Origin and functional consequences of the complex I defect in Parkinson's disease." ANNALS OF NEUROLOGY, vol. 40, 1996, pages 663-671, XP002052334 see abstract see page 664, column 1, paragraph 3 - page 665, column 1, paragraph 3; figure 1 ---</p>	15-20
A	<p>ANDERSON S ET AL.: "Sequence and organization of the human mitochondrial genome." NATURE, vol. 290, 1981, pages 457-465, XP002052335 cited in the application see the whole document -----</p>	

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 97/19023

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
see FURTHER INFORMATION sheet PCT/ISA/210
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☒ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Remark : Although claims 21-26
are directed to a method of treatment of
the human/animal body , the search has been carried out and based on the
alleged effects of the compound/composition.

INVITATION TO PAY ADDITIONAL FEES

International application No.

PCT/US 97/19023

1. Claims: 1-3,7,8,19,20 (complete), 6,10-18,21-29 (partial)

Invention 1: Method to identify mutations within human mitochondrial ATP synthase genes 6 or 8 and isolation of nucleotide sequences therefore, method to inhibit transcription or translation and to evaluate a compound useful in diagnosis and treatment, as well as method for treating an individual afflicted with diabetes.

2. Claims: 4,5,9 (complete), 6,10-18,21-29 (partial)

Invention 2: Method to identify mutations within human mitochondrial tRNA(Lys) and isolation of nucleotide sequences therefore, method to inhibit transcription or translation and to evaluate a compound useful in diagnosis and treatment, as well as method for treating an individual afflicted with diabetes.

Although the application contains two different inventions, a complete search was carried out by the ISA, taken into account the balance between the levying of additional search fees and the necessary additional search effort.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 97/19023

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9119815 A	26-12-91	US 5296349 A	22-03-94
		AU 8293491 A	07-01-92
WO 9526973 A	12-10-95	US 5565323 A	15-10-96
		AU 2204295 A	23-10-95
		CA 2186636 A	12-10-95
		EP 0751951 A	08-01-97
		FI 963884 A	26-11-96
		NO 964073 A	29-11-96

Form PCT/ISA/210 (patent family annex) (July 1992)